VERIFICATION OF TRANSLATION

I, <u>Maiko Harada</u>

of <u>6th Fl., Kantetsu Tsukuba-Science-City Bldg. 1-1-1, Oroshi-machi, Tsuchiura, Ibaraki,</u>
<u>JAPAN</u>

declare as follows:

1. That I am well acquainted with both the English and <u>Japanese</u> languages, and

2. That the attached document is a true and correct translation of a certified copy of the following application, which was made by me to the best of my knowledge and belief of.

    Japanese Patent Application No. <u>2003-415760</u>
    Entitled: "Methods for enhancing antibody activity"
    Filed on December 12, 2003

June 5, 2008
      Date

Maiko Harada
      Maiko Harada

【Name of Document】 APPLICATION FOR PATENT

【Identification Number】 C1-A0321

【Filing Date】 December 12, 2003

【Addressee】 Esq. Commissioner of the Patent Office

【Inventor】

　【Address】　　　c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 153-2, Nagai, Niihari-mura, Niihari-gun, IBARAKI

　【Name】　　　OHTOMO Toshihiko

【Inventor】

　【Address】　　　c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 135, Komakado 1-chome, Gotenba-shi, SHIZUOKA

　【Name】　　　YABUTA Naohiro

【Inventor】

　【Address】　　　c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 153-2, Nagai, Niihari-mura, Niihari-gun, IBARAKI

　【Name】　　　TSUNODA Hiroyuki

【Inventor】

　【Address】　　　c/o CHUGAI SEIYAKU KABUSHIKI KAISHA, 135, Komakado 1-chome, Gotenba-shi, SHIZUOKA

　【Name】　　　TSUCHIYA Masayuki

【Applicant】

　【Identification Number】　　　000003311

　【Name or Appellation】　　　CHUGAI SEIYAKU KABUSHIKI KAISHA

【Attorney】

　【Identification Number】　　　100102978

　【Patent Attorney】

　【Name or Appellation】　　　SHIMIZU Hatsushi

【Nominated Attorney】

　【Identification Number】　　　100108774

【Patent Attorney】

　　【Name or Appellation】　　　　HASHIMOTO Kazunori

【Payment】

　　【Registration Number】　　　041092

　　【Amount】　　　　　　　　21000 yen

【List of Attached Documents】

　　【Name of Document】　Claims　　　　1

　　【Name of Document】　Specification　　1

　　【Name of Document】　Drawings　　　1

　　【Name of Document】　Abstract　　　1

【General Power of Attorney Number】　0216136

[Document Name] Claims

[Claim 1]　A method for enhancing the activity of an antibody, which comprises making the antibody into a single-chain polypeptide comprising two or more light chain variable regions and two or more heavy chain variable regions linked via linkers.

[Claim 2]　A method for enhancing the activity of an antibody, which comprises linking a first polypeptide to a second polypeptide by a linker, wherein the first polypeptide comprises the antibody's heavy chain variable region and light chain variable region and the second polypeptide comprises the antibody's heavy chain variable region and light chain variable region.

[Claim 3]　A method for enhancing the activity of an antibody, which comprises converting the antibody into an sc(Fv)2.

[Claim 4]　The method according to any one of claims 1 to 3, wherein the activity is an agonistic activity.

[Claim 5]　The method according to any one of claims 1 to 4, wherein the linker is a peptide linker.

[Claim 6]　The method according to claim 5, wherein the length of the peptide linker is 5 to 30 amino acids.

[Claim 7]　The method according to claim 6, wherein the length of the peptide linker is 12 to 18 amino acids.

[Claim 8]　The method according to claim 7, wherein the length of the peptide linker is 15 amino acids.

[Claim 9]　An antibody whose activity has been enhanced by the method according to any one of claims 1 to 8.

[Claim 10]　A method for producing the antibody of claim 9, which comprises:

(a) preparing a DNA that encodes two or more antibody heavy chain variable regions, two or more antibody light chain variable regions, and peptide linkers linking each of the variable regions;

(b) constructing a vector comprising the DNA;

(c) introducing the vector into a host cell; and

(d) culturing the host cell.

[Claim 11]　The production method according to claim 10, wherein the DNA encodes two heavy chain variable regions, two light chain variable regions, and three peptide linkers.

[Claim 12]　The production method according to claim 11, wherein the DNA is encoded in the order of: heavy chain variable region, peptide linker, light chain variable region, peptide linker, heavy chain variable region, peptide linker, and light chain variable region.

[Document Name] Specification

[Title of Invention] METHOD OF REINFORCING ANTIBODY ACTIVITY

[Field of the Invention]

[0001]

The present invention relates to methods for enhancing antibody activity.

[Background of the Invention]

5   [0002]

Antibodies receive attention as pharmaceuticals due to their high stability and low antigenicity in blood.   Of these, agonist antibodies which are capable of recognizing cell surface-expressed proteins such as receptors, and thereby induce specific reactions in cells are considered to be useful as pharmaceuticals.   Several agonist antibodies such as agonist

10   antibodies against erythropoietin receptor (see Non-patent Document 1), thrombopoietin receptor or CD47 (see Patent Documents 1 and 2) have been reported.

[0003]

The agonistic activities of these agonist antibodies have been determined by various assay methods; however, the activities are all weaker compared with natural ligands.   For

15   example, in order for an agonist antibody to exert its agonistic activity against the thrombopoietin receptor, which belongs to the cytokine receptor family, it is essential that the TPO receptor is first dimerized and placed at a distance appropriate for signal transduction. Since the antibody molecules are divalent, they can easily dimerize the receptor.   However, since the antibodies are large molecules with a molecular weight of approximately 150kD, they

20   have a low structural flexibility.   Expectantly, it would be difficult for bound receptor to provide the optimal distance for signal transduction and to exhibit a sufficient agonistic activity.

[0004]

[Patent Document 1]   WO 02/33072

[Patent Document 2]   WO 02/33073

25   [Non-patent Document 1]   Elliott S *et al.*, J. Biol. Chem., 1996, Vol. 271(40), p. 24691-24697

[Disclosure of the Invention]

[Problems to Be Solved by the Invention]

[0005]

The present invention was achieved in view of the circumstances described above.   An

30   objective of the present invention is to provide methods for enhancing antibody activity. Specifically, the objective is to provide methods for enhancing antibody activity, which comprise preparing a single-chain polypeptide in which two or more light chain variable regions are linked to two or more heavy chain variable regions via linkers.

[Means for Solving the Problems]

35   [0006]

A minibody, specifically, a diabody or an sc(Fv)2 that has a molecular weight of about 60 kD, which is less than half of a full-size antibody, is predicted to have a relatively high degree

of structural flexibility, and is thought to dimerize a receptor more efficiently or as efficiently as a ligand, thereby exhibiting higher activity.

[0007]

The present inventors prepared and purified an anti-human Mpl antibody, and then constructed a single-chain antibody from the anti-human Mpl antibody VB22B using genetic engineering techniques. Further, the inventors constructed an expression vector for the anti-human Mpl antibody sc(Fv)2, and transiently expressed the single-chain antibody in CHO-DG44 cells. Then, the inventors obtained the single-chain anti-human Mpl antibody VB22B sc(Fv)2 from the culture supernatant. As control, an expression vector for an anti-human Mpl diabody was constructed and expressed in COS7 cells, and the VB22B diabody was obtained from the culture supernatant. Both antibodies were evaluated for their TPO-like agonist activities, and the single-chain antibody was found to exhibit a higher agonistic activity. This finding shows that the activity of an antibody can be enhanced by converting the antibody to a single-chain polypeptide comprising two or more heavy chain variable regions and two or more light chain variable regions linked via linkers.

[0008]

Specifically, the present invention relates to methods for enhancing antibody activity, more specifically to:

[1] a method for enhancing the activity of an antibody, which comprises making the antibody into a single-chain polypeptide comprising two or more light chain variable regions and two or more heavy chain variable regions linked via linkers;

[2] a method for enhancing the activity of an antibody, which comprises linking a first polypeptide to a second polypeptide by a linker, wherein the first polypeptide comprises the antibody's heavy chain variable region and light chain variable region and the second polypeptide comprises the antibody's heavy chain variable region and light chain variable region;

[3] a method for enhancing the activity of an antibody, which comprises converting the antibody into an sc(Fv)2;

[4] the method according to any one of [1] to [3], wherein the activity is an agonistic activity;

[5] the method according to any one [1] to [4], wherein the linker is a peptide linker;

[6] the method according to [5], wherein the length of the peptide linker is 5 to 30 amino acids;

[7] the method according to [6], wherein the length of the peptide linker is 12 to 18 amino acids;

[8] the method according to [7], wherein the length of the peptide linker is 15 amino acids;

[9] an antibody whose activity has been enhanced by the method according to any one of [1] to [8];

[10] a method for producing the antibody of [9], which comprises:

(a) preparing a DNA that encodes two or more antibody heavy chain variable regions, two or more antibody light chain variable regions, and peptide linkers linking each of the variable regions;

(b) constructing a vector comprising the DNA,

(c) introducing the vector into a host cell, and

(d) culturing the host cell;

[11] the production method according to [10], wherein the DNA encodes two heavy chain variable regions, two light chain variable regions, and three peptide linkers; and

[12] the production method according to [11], wherein the DNA is encoded in the order of: heavy chain variable region, peptide linker, light chain variable region, peptide linker, heavy chain variable region, peptide linker, and light chain variable region.

[Effects of the Invention]

[0009]

According to the present invention, even if a full-size antibody exhibits low or no agonistic activity, the activity of the antibody can be enhanced by reducing its molecular weight, specifically, by converting the antibody to a diabody or sc(Fv)2. Thus, full-size antibodies, whose development into pharmaceuticals has been conventionally difficult due to their low activities, can be developed into pharmaceuticals if converted to minibodies. In addition, the manufacturing cost can be reduced if specific activity is improved. Furthermore, since minibodies do not bind sugar chains, various expression systems, such as animal cells, yeast, and *E. coli*, can be easily utilized for the preparation of recombinant minibodies.

[0010]

[Mode for Carrying Out the Invention]

The present invention provides methods for enhancing antibody activity, which comprise converting the antibody into a single-chain polypeptide comprising two or more light chain variable regions and two or more heavy chain variable regions linked via linkers. The antibodies of the present invention, whose activity is to be enhanced, may be any kinds of antibodies including antibodies derived from any kinds of animals such as mouse, human, rat, rabbit, and camel. Furthermore, the antibodies may be any antibodies including, for example, altered antibodies that contain amino acid substitutions, such as chimeric antibodies and humanized antibodies, as well as antibodies modified by linking with various molecules, antibody fragments, and antibodies with modified sugar chains.

In the present invention, an antibody whose activity is enhanced may be a whole antibody or minibody such as diabody.

[0011]

The single-chain polypeptides of the present invention include, for example, single-chain polypeptides in which a first polypeptide comprising antibody heavy chain and light chain variable regions and a second polypeptide comprising antibody heavy chain and light chain variable regions are linked via linkers.

The first polypeptide comprising antibody heavy chain and light chain variable regions may be the same as or different from the second polypeptide comprising heavy chain and light chain variable regions. If the first and second polypeptides are different, the antibody may be an antibody that recognizes one antigen or epitope, or a bispecific antibody recognizing different antigens or epitopes.

[0012]

An exemplary polypeptide comprising antibody heavy chain and light chain variable regions is scFv (single-chain Fv). Thus, sc(Fv)2 is an example of a single-chain polypeptide, in which a first polypeptide comprising antibody heavy chain and light chain variable regions and a second polypeptide comprising antibody heavy chain and light chain variable regions are linked via linkers. sc(Fv)2 is a single-chain polypeptide antibody that comprises two heavy chain variable regions and two light chain variable regions linked via linkers (Hudson *et al*, J Immunol. Methods 1999 ; 231:177-189).

[0013]

For sc(Fv)2, the two heavy chain variable regions (VH) and the two light chain variable regions (VL) can be linked in any order without particular limitation. Examples of the arrangement are listed below.

[VH]- linker- [VL] -linker - [VH] - linker - [VL]

[VL] - linker - [VH] -linker- [VH] - linker - [VL]

[VH] -linker- [VL] -linker - [VL] - linker - [VH]

[VH] -linker- [VH] -linker - [VL] - linker- [VL]

[VL] -linker - [VL] -linker- [VH] - linker- [VH]

[VL] -linker - [VH] -linker- [VL] - linker - [VH]

In the present invention, sc (Fv) 2 is preferably arranged in the order of: [VH] linker [VL] linker [VH] linker [VL].

[0014]

The amino acid sequences of the heavy chain variable regions or light chain variable regions may contain substitutions, deletions, additions and/or insertions. Furthermore, the antibodies may also lack portions of the heavy chain variable regions and/or light chain variable regions, and an alternative polypeptide(s) may be added thereto, as long as they have antigen-binding ability. In addition, the variable regions may be chimerized or humanized.

Alterations of amino acid sequences, such as amino acid substitutions, deletions,

additions, and/or insertions, humanization and chimerization, can be achieved after the activity is enhanced by the methods of the present invention.   Alternatively, activity enhancement by the methods of the present invention may be done after amino acid sequences are altered.

Chimeric antibodies are antibodies prepared by combining sequences derived from
5   different animal species, and include for example, antibodies comprising the heavy chain and light chain variable regions of a murine antibody, and the heavy chain and light chain constant regions of a human antibody.   Chimeric antibodies can be prepared by known methods.   For example, a DNA encoding the V region of an antibody is linked to a DNA encoding the C region of a human antibody, and the construct is inserted into an expression vector and introduced into a
10   host to produce chimeric antibodies.
[0015]

Humanized antibodies are also referred to as "reshaped human antibodies".   Such a humanized antibody is obtained by transferring the complementarity-determining region (CDR) of an antibody derived from a non-human mammal, for example mouse, to the
15   complementarity-determining region of a human antibody, and the general gene recombination procedure for this is also known (see European Patent Application No. 125023 and WO 96/02576).
[0016]

Specifically, a DNA sequence designed to link a murine antibody CDR to the
20   framework region (FR) of a human antibody can be synthesized by PCR, using primers prepared from several oligonucleotides containing overlapping portions of both CDR and FR terminal regions (see methods described in WO 98/13388).

The human antibody framework region to be linked by CDR is selected in order to form a favorable antigen-binding site in the complementarity-determining region.   Amino acids of
25   the framework region in the antibody variable region may be substituted, as necessary, for the complementarity-determining region of the reshaped human antibody to form a suitable antigen-binding site (Sato, K. *et al.*, 1993, Cancer Res. 53, 851-856).
[0017]

The constant region of a human antibody is used as the C region of a chimeric antibody
30   or humanized antibody.   For example, Cγ1, Cγ2, Cγ3, and Cγ4 can be used as the H chain, and Cκ and Cλ can be used as the L chain.   The human antibody C region may be modified to improve the antibody or the stability of the antibody production.

Generally, chimeric antibodies comprise the variable region of an antibody from a non-human mammal and the constant region derived from a human antibody.   On the other
35   hand, humanized antibodies comprise the complementarity-determining region of an antibody from a non-human mammal, and the framework region and C region derived from a human antibody.

[0018]

     In addition, amino acids of the variable region (for example, FR) and the constant region of a prepared chimeric antibody or humanized antibody may be substituted with other amino acids. The sequence of the antibody variable region may be a sequence of a variable

5   region of any known antibody, or an antibody sequence prepared using an appropriate antigen by methods known to those skilled in the art. Specifically, the sequence can be determined by, for example, the following procedure. Immunized animals such as mice are immunized with an antigen by a conventional immunization method. The immunocytes obtained are fused with known parental cells by a conventional cell fusion method and screened for monoclonal

10   antibody-producing cells (hybridomas) by a conventional screening method. The antigen can be prepared by known methods. Hybridomas can be prepared, for example, by the method of Milstein *et al.* (Kohler, G. and Milstein, C., Methods Enzymol. (1981) 73:3-46). If the antigen has a low immunogenicity, it may be linked with an immunogenic macromolecule such as albumin, and then used for immunization. Then, the antibody variable region (V region) cDNA

15   is synthesized from the hybridoma mRNA using reverse transcriptase, and the obtained cDNA sequence is determined by known methods.

[0019]

     Meanwhile, methods for preparing human antibodies are also well known. Desired human antibodies with binding activity can be obtained by, for example, sensitizing human

20   lymphocytes in vitro and fusing the sensitized lymphocytes with human myeloma cells that are capable of permanent cell division (see Japanese Patent Application Kokoku Publication No. (JP-B) H1-59878 (examined, approved Japanese patent application published for opposition)). Alternatively, an antigen is administered to a transgenic animal having a repertoire of all human antibody genes, and antibody-producing cells are produced and immortalized to obtain human

25   antibodies against the antigen (see International Patent Application WO 94/25585, WO 93/12227, WO92/03918, and WO 94/02602).

[0020]

     In the present invention, arbitrary peptide linkers that can be introduced by genetic engineering, or synthetic linkers (for example, linkers disclosed in "Protein Engineering, 9(3),

30   299-305, 1996"), can be used as linkers to link a heavy chain variable region and a light chain variable region.

[0021]

     There are no limitations as to the length of the peptide linkers. The length can be appropriately selected by those skilled in the art according to the purpose, and is typically 1 to

35   100 amino acids, preferably 5 to 30 amino acids, and more preferably 12 to 18 amino acids (e.g., 15 amino acids).

[0022]

Amino acid sequences of such peptide linkers include, for example:

Ser

Gly・Ser

Gly・Gly・Ser

5 Ser・Gly・Gly

Gly・Gly・Gly・Ser

Ser・Gly・Gly・Gly

Gly・Gly・Gly・Gly・Ser

Ser・Gly・Gly・Gly・Gly

10 Gly・Gly・Gly・Gly・Gly・Ser

Ser・Gly・Gly・Gly・Gly・Gly

Gly・Gly・Gly・Gly・Gly・Gly・Ser

Ser・Gly・Gly・Gly・Gly・Gly・Gly

(Gly・Gly・Gly・Gly・Ser)n

15 (Ser・Gly・Gly・Gly・Gly)n

where n is an integer of 1 or larger.

[0023]

Synthetic linkers (chemical crosslinking agents) include crosslinking agents that are routinely used to crosslink peptides, for example, N-hydroxy succinimide (NHS), disuccinimidyl

20 suberate (DSS), bis(sulfosuccinimidyl) suberate (BS^3), dithiobis(succinimidyl propionate) (DSP), dithiobis(sulfosuccinimidyl propionate) (DTSSP), ethylene glycol bis(succinimidyl succinate) (EGS), ethylene glycol bis(sulfosuccinimidyl succinate) (sulfo-EGS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo-DST), bis[2-(succinimidoxycarbonyloxy)ethyl] sulfone (BSOCOES), and bis[2-(sulfosuccinimidoxycarbonyloxy)ethyl] sulfone

25 (sulfo-BSOCOES). These crosslinking agents are commercially available..

The present invention also provides antibodies whose activities have been improved by methods described above.

[0024]

The present invention also provides methods for producing antibodies, which comprise:

30 (a) preparing a DNA encoding two or more antibody heavy chain variable regions, two or more antibody light chain variable regions, and peptide linkers to be used for linking each of the variable regions;

(b) preparing a vector comprising the DNA;

(c) introducing the vector into a host cell; and

35 (d) culturing the host cell.

In these methods, first, a DNA that encodes two or more antibody heavy chain variable regions, two or more antibody light chain variable regions, and peptide linkers that link the

variable regions is prepared.   Such DNA includes, for example, DNA encoding two heavy chain variable region (VH) and two light chain variable region (VL), and three peptide linkers.   A preferred DNA is an sc (Fv)2-encoding DNA.

[0025]

5   The two VHs and two VLs can be linked in any order without particular limitation. Examples of the arrangement are listed below.

[VH] -linker- [VL]- linker- [VH] -linker- [VL]

[VL] -linker - [VH] -linker- [VH] -linker - [VL]

[VH] - linker- [VL] - linker- [VL] - linker v[VH]

10   [VH] -linker- [VH] -linker- [VL] -linker - [VL]

[VL] -linker- [VL] -linker- [VH] -linker- [VH]

[VL] -linker- [VH] - linker- [VL] - linker- [VH]

In the present invention, a preferred arrangement is: [VH] - linker- [VL] -linker- [VH] -linker- [VL].

15   [0026]

The amino acid sequences of the heavy chain variable regions or light chain variable regions may contain substitutions, deletions, additions and/or insertions.   Furthermore, the antibody may also lack portions of the heavy chain variable regions or/and light chain variable regions, as long as it has an antigen-binding activity.   In addition, the variable regions may be

20   chimerized or humanized.

[0027]

The present invention further includes construction of vectors containing the DNAs mentioned above.

When *E. coli* is used as a host, there is no particular limitation as to the type of vector of the

25   present invention, as long as the vector contains an "ori" responsible for its replication in *E. coli* and a marker gene.   The "ori" ensures the amplification and mass production of the vector in *E. coli* (for example, JM109, DH5α, HB101, and XL1Blue).   The marker gene is used to select the *E. coli* transformants (for example, a drug resistance gene selected by an appropriate drug such as ampicillin, tetracycline, kanamycin, and chloramphenicol).   The vectors include,

30   for example, M13 vectors, pUC vectors, pBR322, pBluescript, and pCR-Script.   In addition to the above vectors, for example, pGEM-T, pDIRECT, and pT7 can also be used for the subcloning and excision of cDNAs.

[0028]

In particular, expression vectors are useful as vectors of the present invention.   When

35   an expression vector is expressed, for example, in *E. coli*, it should have the above characteristics in order to be amplified in *E. coli*.   Additionally, when *E. coli*, such as JM109, DH5α, HB101, or XL1-Blue are used as the host cell, the vector preferably has a promoter, for example, lacZ

promoter (Ward *et al.* (1989) Nature 341:544-546; (1992) FASEB J. 6:2422-2427), araB promoter (Better *et al.* (1988) Science 240:1041-1043), or T7 promoter, that allows efficient expression of the desired gene in *E. coli*.   Other examples of the vectors include pGEX-5X-1 (Pharmacia), "QIAexpress system" (QIAGEN), pEGFP, and pET (where BL21, a strain

5   expressing T7 RNA polymerase, is preferably used as the host).

[0029]

Furthermore, the vectors may comprise a signal sequence for polypeptide secretion. When producing polypeptides into the periplasm of *E. coli*, the pelB signal sequence (Lei, S. P. *et al.* J. Bacteriol. 169:4379 (1987)) may be used as a signal sequence for polypeptide secretion.

10   For example, calcium chloride methods or electroporation methods may be used to introduce the vector into a host cell.

[0030]

In addition to *E. coli*, expression vectors derived from mammals (e.g., pCDNA3 (Invitrogen), pEGF-BOS (Nucleic Acids Res. (1990) 18(17):5322), pEF, pCDM8), insect cells

15   (e.g., "Bac-to-BAC baculovirus expression system" (GIBCO-BRL), pBacPAK8), plants (e.g., pMH1, pMH2), animal viruses (e.g., pHSV, pMV, pAdexLcw), retroviruses (e.g., pZIPneo), yeasts (e.g., "Pichia Expression Kit" (Invitrogen), pNV11, SP-Q01), and *Bacillus subtilis* (e.g., pPL608, pKTH50) may also be used as a vector of the present invention.

[0031]

20   In order to express proteins in animal cells such as CHO, COS, and NIH3T3 cells, the vector preferably has a promoter necessary for expression in such cells, for example, an SV40 promoter (Mulligan *et al.* (1979) Nature 277:108), MMLV-LTR promoter, EF1α promoter (Mizushima *et al.* (1990) Nucleic Acids Res. 18:5322), CMV promoter, etc.   It is even more preferable that the vector also carries a marker gene for selecting transformants (for example, a

25   drug-resistance gene selected by a drug such as neomycin and G418).   Examples of vectors with such characteristics include pMAM, pDR2, pBK-RSV, pBK-CMV, pOPRSV, and pOP13, and such.

[0032]

In addition, to stably express a gene and amplify the gene copy number in cells, CHO

30   cells that are defective in the nucleic acid synthesis pathway are introduced with a vector containing a DHFR gene (for example, pCHOI) to compensate for the defect, and the copy number is amplified using methotrexate (MTX).   Alternatively, a COS cell, which carries an SV40 T antigen-expressing gene on its chromosome, can be transformed with a vector containing the SV40 replication origin (for example, pcD) for transient gene expression.   The

35   replication origin may be derived from polyoma virus, adenovirus, bovine papilloma virus (BPV), and such.   Furthermore, to increase the gene copy number in host cells, the expression vector may contain, as a selection marker, aminoglycoside transferase (APH) gene, thymidine

kinase (TK) gene, *E. coli* xanthine guanine phosphoribosyl transferase (Ecogpt) gene, dihydrofolate reductase (dhfr) gene, and such.

[0033]

In the present invention, next, the vector is introduced into a host cell. The host cells into which the vector is introduced are not particularly limited, for example, *E. coli* and various animal cells are available for this purpose. The host cells may be used, for example, as a production system to produce and express polypeptides comprising the two or more antibody heavy chain variable regions and the two or more antibody light chain variable regions, and peptide linkers linking each of the variable regions in the present invention. *In vitro* and *in vivo* production systems are available for polypeptide production systems. Production systems that use eukaryotic cells or prokaryotic cells are examples of *in vitro* production systems.

[0034]

Eukaryotic cells that can be used are, for example, animal cells, plant cells, and fungal cells. Known animal cells include: mammalian cells, for example, CHO (J. Exp. Med. (1995)108, 945), COS, 3T3, myeloma, BHK (baby hamster kidney), HeLa, Vero, amphibian cells such as *Xenopus laevis* oocytes (Valle, *et al.* (1981) Nature 291, 358-340), or insect cells (e.g., Sf9, Sf21, and Tn5). In the present invention, CHO-DG44, CHO-DXB11, COS7 cells, and BHK can be suitably used. Among animal cells, CHO cells are particularly favorable for large-scale expression. Vectors can be introduced into a host cell by, for example, calcium phosphate methods, the DEAE-dextran methods, methods using cationic liposome DOTAP (Boehringer-Mannheim), electroporation methods, lipofection methods.

[0035]

Plant cells include, for example, Nicotiana tabacum-derived cells known as a protein production system. Calluses may be cultured from these cells. Known fungal cells include yeast cells, for example, genus Saccharomyces such as Saccharomyces cerevisiae and Saccharomyces pombe; and filamentous fungi, for example, genus Aspergillus such as Aspergillus niger.

Bacterial cells can be used in the prokaryotic production systems. Examples of bacterial cells include *E. coli* (for example, JM109, DH5α, HB101 and such); and Bacillus subtilis.

[0036]

Next, the above host cells are cultured. Antibodies can be obtained by transforming the cells with a DNA of interest and *in vitro* culturing of these transformants. Transformants can be cultured using known methods. For example, DMEM, MEM, RPMI 1640, or IMDM may be used as the culture medium for animal cells, and may be used with or without serum supplements such as FBS or fetal calf serum (FCS). Serum-free cultures are also acceptable. The preferred pH is about 6 to 8 during the course of culturing. Incubation is carried out

typically at a temperature of about 30 to 40°C for about 15 to 200 hours. Medium is exchanged, aerated, or agitated, as necessary.

[0037]

On the other hand, production systems using animal or plant hosts may be used as
5   systems for producing polypeptides *in vivo*.   For example, a DNA of interest is introduced into an animal or plant and the polypeptide is produced in the body of the animal or plant and then recovered.   The "hosts" of the present invention includes such animals and plants.

[0038]

Animals to be used for the production system include mammals or insects.   Mammals
10   such as goats, pigs, sheep, mice, and cattle may be used (Vicki Glaser SPECTRUM Biotechnology Applications (1993)).   Alternatively, the mammals may be transgenic animals.

[0039]

For example, a DNA of interest is prepared as a fusion gene with a gene encoding a polypeptide specifically produced in milk, such as the goat β-casein gene.   DNA fragments
15   containing the fusion gene are injected into goat embryos, which are then introduced back to female goats.   The desired protein can be obtained from milk produced by the transgenic goats, which are born from the goats that received the embryos, or from their offspring.   Appropriate hormones may be administered to increase the volume of milk containing the protein produced by the transgenic goats (Ebert, K.M. *et al.*, Bio/Technology 12, 699-702 (1994)).

20   [0040]

Insects such as silkworms, may also be used.   Baculoviruses carrying a DNA encoding a protein of interest can be used to infect silkworms, and the antibody of interest can be obtained from the body fluids (Susumu, M. *et al.*, Nature 315, 592-594 (1985)).

[0041]

25   Plants used in the production system include, for example, tobacco.   When tobacco is used, a DNA encoding an antibody of interest is inserted into a plant expression vector, for example, pMON 530, and then the vector is introduced into a bacterium, such as Agrobacterium tumefaciens.   The bacteria are then used to infect tobacco such as Nicotiana tabacum, and the desired antibodies can be recovered from the leaves (Julian K.-C. Ma *et al.*, Eur. J. Immunol. 24,
30   131-138 (1994)).

[0042]

The resulting antibody may be isolated from the inside or outside (such as the medium) of host cells, and purified as a substantially pure and homogenous antibody.   Methods are not limited to any specific method and any standard method for isolating and purifying antibodies
35   may be used.   Antibodies may be isolated and purified, by selecting an appropriate combination of, for example, chromatographic columns, filtration, ultrafiltration, salting out, solvent precipitation, solvent extraction, distillation, immunoprecipitation, SDS-polyacrylamide gel

electrophoresis, isoelectric focusing, dialysis, recrystallization, and others.

[0043]

Chromatographies include, for example, affinity chromatographies, ion exchange chromatographies, hydrophobic chromatographies, gel filtrations, reverse-phase

5   chromatographies, and adsorption chromatographies (Strategies for Protein Purification and Characterization: A Laboratory Course Manual. Ed Daniel R. Marshak *et al.*, Cold Spring Harbor Laboratory Press, 1996).   These chromatographies can be carried out using liquid phase chromatographies such as HPLC and FPLC.   Examples of the affinity chromatography columns include protein A columns and protein G columns.   Examples of the proteins A columns include

10   Hyper D, POROS, and Sepharose F. F. (Pharmacia).

[0044]

An antibody can be modified freely and peptide portions deleted by treating the antibody with an appropriate protein modifying enzyme before or after antibody purification. Such protein modifying enzymes include, for example, trypsins, chymotrypsins, lysyl

15   endopeptidases, protein kinases, and glucosidases.

[0045]

The antibody activities to be enhanced in the present invention include, but are not limited to, binding activity, neutralizing activity, cell damaging activity, agonistic activity, antagonistic activity, and enzymatic activity.   However, the activity is preferably an activity that

20   leads to quantitative and/or qualitative changes or effects in an organism, tissue, cell, protein, DNA, RNA, and such.   In particular, agonistic activity is preferred.

[0046]

The term "agonistic activity" refers to an activity that induces changes in some physiological activities through intracellular signal transduction that results from the binding of

25   an antibody to an antigen such as receptor.   The physiological activities include, for example, proliferation activity, survival activity, differentiation activity, transcriptional activity, membrane transport activity, binding activity, proteolytic activity, phosphorylation/dephosphorylation activity, oxidation/reduction activity, transfer activity, nucleolytic activity, dehydration activity, cell death-inducing activity, and apoptosis-inducing activity, without being limited thereto.

30   [0047]

The antigens of the present invention are not particularly limited, and can be any kind of antigen.   Such antigens include, for example, receptors, cancer antigens, MHC antigens, and differentiation antigens.

[0048]

35   The receptors include, for example, receptors that belong to receptor families such as the hematopoietic factor receptor family, cytokine receptor family, tyrosine kinase receptor family, serine/threonine kinase receptor family, TNF receptor family, G protein-coupled receptor

family, GPI-anchored receptor family, tyrosine phosphatase receptor family, adhesion factor family, and hormone receptor family.   Various references that relate to receptors belonging to these receptor families and their characteristics are available and include, for example, Cooke BA., King RJB., van der Molen HJ. ed. New Comprehensive Biochemistry Vol. 18B "Hormones

5   and their Actions Part II" pp. 1-46 (1988) Elsevier Science Publishers BV., New York, USA; Patthy L. (1990) Cell, 61: 13-14; Ullrich A., *et al.* (1990) Cell, 61: 203-212; Massagul J. (1992) Cell, 69: 1067-1070; Miyajima A., *et al.* (1992) Annu. Rev. Immunol., 10: 295-331; Taga T. and Kishimoto T. (1992) FASEB J., 7: 3387-3396; Fantl WI., *et al.* (1993) Annu. Rev. Biochem., 62: 453-481; Smith CA., *et al.* (1994) Cell, 76: 959-962; Flower DR. (1999) Biochim. Biophys. Acta,

10   1422: 207-234; and M. Miyasaka ed., Cell Technology, supplementary volume, Handbook series, "Handbook for Adhesion Factors" (1994) (Shujunsha, Tokyo, Japan).
[0049]
　　　　　Specifically, receptors belonging to the above-described receptor families include, for example, the following human and mouse receptors: erythropoietin (EPO) receptors, granulocyte

15   colony-stimulating factor (G-CSF) receptors, thrombopoietin (TPO) receptors, insulin receptors, Flt-3 ligand receptors, platelet-derived growth factor (PDGF) receptors, interferon (IFN)-α and $-\beta$ receptors, leptin receptors, growth hormone (GH) receptors, interleukin (IL)-10 receptors, insulin-like growth factor (IGF)-I receptors, leukemia inhibitory factor (LIF) receptors, and ciliary neurotrophic factor (CNTF) receptors (hEPOR: Simon, S. *et al.* (1990) Blood 76, 31-35.;

20   mEPOR: D'Andrea, AD. *et al.* (1989) Cell 57, 277-285; hG-CSFR: Fukunaga, R. *et al.* (1990) Proc. Natl. Acad. Sci. USA. 87, 8702-8706; mG-CSFR: Fukunaga, R. *et al.* (1990) Cell 61, 341-350; hTPOR: Vigon, I. *et al.* (1992) 89, 5640-5644; mTPOR: Skoda, RC. *et al.* (1993) 12, 2645-2653; hInsR: Ullrich, A. *et al.* (1985) Nature 313, 756-761; hFlt-3: Small, D. *et al.* (1994) Proc. Natl. Acad. Sci. USA. 91, 459-463; hPDGFR: Gronwald, RGK. *et al.* (1988) Proc. Natl.

25   Acad. Sci. USA. 85, 3435-3439; hIFN α/βR: Uze, G. *et al.* (1990) Cell 60, 225-234; and Novick, D. *et al.* (1994) Cell 77, 391-400).
[0050]
　　　　　Cancer antigens are antigens expressed as a result of malignant cell alteration, and are also referred to as "cancer-specific antigens".   Aberrant sugar chains that are presented on cell

30   surface and on protein molecules due to malignant cell transformation may also serve as cancer antigens, and are referred to as, in particular, "cancer-related carbohydrate antigens".   Cancer antigens include, for example, CA19-9, CA15-3, and sialyl SSEA-1 (SLX).
[0051]
　　　　　MHC antigens are broadly divided into MHC class I and II antigens.   Class I MHC

35   antigens include HLA-A, -B, -C, -E, -F, -G, and –H, and Class II MHC antigens include HLA-DR, -DQ, and -DP.
　　　　　Differentiation antigens include CD1, CD2, CD3, CD4, CD5, CD6, CD7, CD8, CD10,

CD11a, CD11b, CD11c, CD13, CD14, CD15s, CD16, CD18, CD19, CD20, CD21, CD23, CD25, CD28, CD29, CD30, CD32, CD33, CD34, CD35, CD38, CD40, CD41a, CD41b, CD42a, CD42b, CD43, CD44, CD45, CD45RO, CD48, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD51, CD54, CD55, CD56, CD57, CD58, CD61, CD62E, CD62L, CD62P, CD64, CD69, CD71, CD73,

5   CD95, CD102, CD106, CD122, CD126, and CDw130.

[0052]

There is no limitation as to the type of detection indicators to be used for determining activity changes, as long as the indicator can monitor quantitative and/or qualitative changes. For example, it is possible to use cell-free assay indicators, cell-based assay indicators,

10   tissue-based assay indicators, and *in vivo* assay indicators.

[0053]

Indicators that can be used in cell-free assays include enzymatic reactions, quantitative and/or qualitative changes in proteins, DNAs, or RNAs. Such enzymatic reactions include, for example, amino acid transfers, sugar transfers, dehydrations, dehydrogenations, and substrate

15   cleavages. Alternatively, protein phosphorylations, dephosphorylations, dimerizations, multimerizations, hydrolyses, dissociations and such; DNA or RNA amplifications, cleavages, and extensions can be used as the indicator in cell-free assays. For example, protein phosphorylations downstream of a signal transduction pathway may be used as a detection indicator.

20   [0054]

Alterations in cell phenotype, for example, quantitative and/or qualitative alterations in products, alterations in growth activity, alterations in cell number, morphological alterations, or alterations in cellular properties, can be used as the indicator in cell-based assays. The products include, for example, secretory proteins, surface antigens, intracellular proteins, and mRNAs.

25   The morphological alterations include, for example, alterations in dendrite formation and/or dendrite number, alteration in cell flatness, alteration in cell elongation/axial ratio, alterations in cell size, alterations in intracellular structure, heterogeneity/homogeneity of cell populations, and alterations in cell density. Such morphological alterations can be observed under a microscope. Cellular properties to be used as the indicator include anchor dependency, cytokine-dependent

30   response, hormone dependency, drug resistance, cell motility, cell migration activity, pulsatory activity, and alteration in intracellular substances. Cell motility includes cell infiltration activity and cell migration activity. The alterations in intracellular substances include, for example, alterations in enzyme activity, mRNA levels, levels of intracellular signaling molecules such as Ca^{2+} and cAMP, and intracellular protein levels. When a cell membrane receptor is used,

35   alterations in the cell proliferating activity induced by receptor stimulation can be used as the indicator.

[0055]

The indicators to be used in tissue-based assays include functional alterations adequate for the subject tissue.   In *in vivo* assays, alterations in tissue weight, alterations in the blood system (for example, alterations in blood cell counts, protein contents, or enzyme activities), alterations in electrolyte levels, and alterations in the circulating system (for example, alterations

5   in blood pressure or heart rate).

[0056]

The methods for measuring such detection indices are not particularly limited.   For example, absorbance, luminescence, color development, fluorescence, radioactivity, fluorescence polarization, surface plasmon resonance signal, time-resolved fluorescence, mass, absorption

10   spectrum, light scattering, and fluorescence resonance energy transfer may be used.   These measurement methods are known to those skilled in the art and may be selected appropriately depending on the purpose.

[0057]

For example, absorption spectra can be obtained by using a conventional photometer,

15   plate reader, or such; luminescence can be measured with a luminometer or such; and fluorescence can be measured with a fluorometer or such.   Mass can be determined with a mass spectrometer.   Radioactivity can be determined with a device such as a gamma counter depending on the type of radiation.   Fluorescence polarization can be measured with BEACON (TaKaRa).   Surface plasmon resonance signals can be obtained with BIACORE.

20   Time-resolved fluorescence, fluorescence resonance energy transfer, or such can be measured with ARVO or such.   Furthermore, a flow cytometer can also be used for measuring.   It is possible to use one of the above methods to measure two or more different types of detection indices.   A greater number of detection indices may also be examined by using two or more measurement methods simultaneously and/or consecutively.   For example, fluorescence and

25   fluorescence resonance energy transfer can be measured at the same time with a fluorometer.

[0058]

In the present invention, the agonistic activity can be determined by assay methods known to those skilled in the art.   For example, the activity can be determined by the measurement methods described in the EXAMPLES, using cell proliferation as an indicator.

30   More specifically, an antibody whose agonistic activity is to be determined is added to cells that proliferate in an agonist-dependent manner, followed by incubation of the cells.   Then, a reagent such as WST-8, which undergoes a chromogenic reaction at a specific wavelength depending on the viable cell count, is added and the absorbance is measured.   The agonistic activity can be determined using the measured absorbance as an indicator.

35   [0059]

Cells that proliferate in an agonist-dependent manner can also be prepared by methods known to those skilled in the art.   For example, when the antigen is a receptor capable of

transducing cell growth signals, cells expressing the receptor may be used. Alternatively, when the antigen is a receptor that cannot transduce signals, a chimeric receptor comprising the intracellular domain of a receptor that transduces cell growth signals and the extracellular domain of a receptor that does not transduce cell growth signals can be prepared for cellular

5   expression. Receptors that transduce cell growth signals include, for example, G-CSF receptors, mpl, neu, GM-CSF receptors, EPO receptors, c-kit, and FLT-3. Cells that can be used to express a receptor include, for example, BaF3, NFS60, FDCP-1, FDCP-2, CTLL-2, DA-1, and KT-3.

[Examples]

10   [0060]

Herein below, the present invention will be specifically described with reference to Examples.

[0061]

[Example 1]   Preparation of anti-human Mpl antibodies

15   1.1 Establishment of Mpl-expressing BaF3 cell lines

BaF3 cell lines expressing the full-length Mpl gene were established to obtain cell lines that proliferate in a TPO-dependent manner. A full-length human Mpl cDNA (Palacios, R. *et al.*, Cell, 41, 727-734 (1985)) (GenBank accession NO. NM_005373) was amplified by PCR. The cDNA was cloned into a pCOS2 expression vector to construct pCOS2-hMplfull. The

20   expression vector pCOS2 was constructed by removing the DHFR gene expression region from pCHOI (Hirata, Y. *et al.*, FEBS Letter, 356, 244-248 (1994)), where the neomycin resistance gene expression region from HEF-VH-gγ1 (Sato, K. *et al.*, Mol Immunol., 31, 371-381 (1994)) is inserted. The cynomolgus monkey Mpl cDNA ((SEQ ID NO: 1) and the amino acid sequence of a protein encoded thereby (SEQ ID NO: 2)) was cloned from total RNA extracted

25   from the bone marrow cells of cynomolgus monkey, using a SMART RACE cDNA Amplification Kit (Clontech). The resulting cynomolgus monkey cDNA was inserted into pCOS2 to construct pCOS2-monkeyMplfull.

[0062]

Each vector (20 μg) prepared as described above was mixed with BaF3 cells (1x 10^7

30   cells/mL) suspended in PBS in Gene Pulser cuvettes. This mixture was then pulsed at 0.33 kV and 950 μFD using a Gene Pulser II (Bio-Rad). The BaF3 cells introduced with the above DNAs by electroporation were added to RPMI 1640 medium (Invitrogen) containing 1 ng/mL mouse interleukin 3 (hereinafter abbreviated as mIL-3; Peprotech), 500 μg/mL Geneticin (Invitrogen), and 10% FBS (Invitrogen), and selected to establish a human Mpl-expressing BaF3

35   cell line (hereinafter abbreviated as "BaF3-human Mpl"), and a monkey Mpl-expressing BaF3 cell line (hereinafter abbreviated as BaF3-monkey Mpl). Following selection, these cells were cultured and maintained in RPMI 1640 containing 1 ng/mL rhTPO (R&D) and 10% FBS.

[0063]

1.2 Establishment of Mpl-expressing CHO cell lines

CHO cell lines expressing the full-length Mpl gene were established to obtain cell lines to be used for assessing binding activity by flow cytometry. First, the DHFR gene expression site from pCHOI was inserted into pCXN2 (Niwa, H. *et al.*, Gene, 108, 193-199 (1991)) at the HindIII site to prepare a pCXND3expression vector. The respective Mpl genes were amplified by PCR using pCOS2-hMplfull, and pCOS2-monkeyMplfull as templates, and primers with a His-tag sequence. The PCR products were cloned into pCXND3 to construct pCXND3-hMpl-His, and pCXND3-monkey Mpl-His, respectively.

[0064]

Vectors thus prepared (25 μg each) were mixed with a PBS suspension of CHO-DG44 cells (1×10^7 cells/mL) in Gene Pulser cuvettes. The mixture was then pulsed at 1.5 kV and 25 μFD using Gene Pulser II (Bio-Rad). The CHO cells introduced with these DNAs by electroporation were added to CHO-S-SFMII medium (Invitrogen) containing 500 μg/mL Geneticin and 1x HT (Invitrogen). A human Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-human Mpl"), and a monkey Mpl-expressing CHO cell line (hereinafter abbreviated as "CHO-monkey Mpl") were established through selection.

[0065]

1.3 Preparation of soluble human Mpl protein

To prepare soluble human Mpl protein, an expression system using insect Sf9 cells for production and secretion of the protein was constructed as described below. A DNA construct encoding the extracellular region of human Mpl (Gln 26 to Trp 491) with a downstream FLAG tag was prepared. The construct was inserted into a pBACSurf-1 Transfer Plasmid (Novagen) between the PstI and SmaI sites to prepare pBACSurf1-hMpl-FLAG. Then, Sf9 cells were transformed with 4 μg of pBACSurf1-hMpl-FLAG using the Bac-N-Blue Transfection Kit (Invitrogen). The culture supernatant was collected after a three-day incubation. Recombinant virus was isolated by plaque assays. The prepared virus stock was used to infect Sf9 cells, and the culture supernatant was collected.

[0066]

Soluble human Mpl protein was purified from the obtained culture supernatant as described below. The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and the adsorbed protein was then eluted with 50 mM Na-phosphate buffer (pH7.2) containing 0.01% (v/v) Tween 20 and 500 mM NaCl. After the eluates were loaded onto a FLAG M2-Agarose (Sigma-Aldrich) for adsorption, the protein adsorbed was eluted with 100 mM glycine-HCl buffer (pH3.5) containing 0.01% (v/v) Tween 20. Immediately after elution, the fraction obtained was neutralized with 1 M Tris-HCl Buffer (pH8.0) and the buffer was exchanged with PBS(-) and 0.01% (v/v) Tween 20 using PD-10

columns (Amersham Biosciences).   The purified soluble Mpl protein was referred to as "shMpl-FLAG".

[0067]

1.4 Preparation of human Mpl-IgG Fc fusion protein

Human fusion protein Mpl-IgG Fc gene was prepared according to the method by Bennett *et al.* (Bennett, B. D. *et al.*, J. Biol. Chem. 266, 23060-23067 (1991)).   A nucleotide sequence encoding the extracellular region of human Mpl (Gln 26 to Trp 491) was linked to a nucleotide sequence encoding the Fc region of human IgG-γ1 (a region downstream of Asp 216). A BstEII sequence (amino acids: Val-Thr) was attached to the junction as a fusion linker between these two regions.   A 19-amino acid signal peptide derived form human IgG H chain variable region was used as the signal sequence.   The resulting human fusion protein Mpl-IgG Fc gene was cloned into pCXND3 to construct pCXND3-hMpl-Fc.

[0068]

The vectors thus prepared (25 μg) was each mixed with a PBS suspension of CHO-DG44 cells (1 x 10^7 cells/mL) in Gene Pulser cuvettes.   The mixture was then pulsed at 1.5 kV and 25 μFD using Gene Pulser II (Bio-Rad).   The CHO cells introduced with the DNA by electroporation were added to CHO-S-SFMII medium containing 500 μg/mL Geneticin and 1x HT (Invitrogen).   shMPL-Fc-expressing CHO cell line (CHO-hMpl-Fc) was then established through selection.

[0069]

Human Mpl-IgG Fc fusion protein was purified from the culture supernatant as described below.   The culture supernatant was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) for adsorption, and then the adsorbed protein were eluted with 50 mM Na-phosphate buffer (pH7.6) containing 0.01% (v/v) Tween 20 and 1 M NaCl.   After the eluates were loaded onto a HiTrap protein G HP column (Amersham Biosciences) for adsorption, the adsorbed protein was eluted with 0.1 M glycine-HCl buffer (pH2.7) containing 150 mM NaCl and 0.01% (v/v) Tween 20.   Immediately after elution, the obtained fraction was neutralized with 1 M Tris-HCl Buffer (pH8.0) and the buffer was exchanged with PBS(-) and 0.01% (v/v) Tween 20 using PD-10 columns (Amersham Biosciences).   The purified soluble Mpl protein was referred to as "hMpl-Fc".

[0070]

1.5 Immunization with shMpl-FLAG or BaF-human Mpl and hybridoma selection

MRL/MpJUmmCrj-lpr/lpr mice (hereinafter abbreviated as "MRL/lpr mice"; purchased from Charles River, Japan) were immunized; the primary immunization was carried out at eight weeks of age.   For every single mouse, an emulsion containing 100 μg of shMPL-FLAG combined with Freud's complete adjuvant (H37 Ra; Beckton Dickinson), was administered subcutaneously as the primary injection.   As a booster injection, an emulsion containing

shMPL-FLAG (50 µg per mouse) combined with Freud's incomplete adjuvant (Beckton Dickinson) was administered subcutaneously.   Three mice which have been immunized six times in total were subjected to a final injection of shMPL-FLAG (50 µg per mouse) through the caudal vein.   Cell fusion was achieved by mixing the mouse myeloma P3-X63Ag8U1 cells

5   (P3U1; purchased from ATCC) and mouse splenocytes using polyethylene glycol 1500 (Roche Diagnostics).   Hybridoma selection in HAT medium began the following day and culture supernatants were obtained.   Screening was carried out by ELISA, using immunoplates with immobilized shMpl-FLAG or hMpl-Fc and the assayed cell proliferation activity of BaF3-hMpl as an index.   Positive clones were isolated as single clones by limiting dilution and then

10   cultured on a large scale.   The culture supernatants were collected.   Anti-human Mpl antibody-producing hybridomas, VB22B, VB16, VB140, and VB45B, were obtained by this method.
[0071]

In addition, Balb/C mice (Charles River Laboratories, Japan) were intraperitoneally
15   administered with 1.0×10^7 cells of BaF-human Mpl for a total of eleven times over a period of one week to five months.   Spleen cells from these mice were fused with mouse myeloma cell P3U1 as described above.   Cell selection was carried out the next day using a HAT medium and screening was performed using as an index the cell proliferation activity of Baf3-hMpl in the culture supernatant.   Positive clones were isolated as single clones by limiting dilution and then

20   cultured on a large scale.   The culture supernatants were collected.   An anti-human Mpl antibody-producing hybridoma, TA136, was obtained by this method.
[0072]
1.6 Analyses of anti-human Mpl antibodies

Antibody concentrations were determined by carrying out a mouse IgG sandwich
25   ELISA using goat anti-mouse IgG (gamma) (ZYMED) and alkaline phosphatase-goat anti-mouse IgG (gamma) (ZYMED), generating a calibration curve by GraphPad Prism (GraphPad Software; USA), and calculating the antibody concentrations from the calibration curve.   Commercially available antibodies of the same isotype were used as standards.
[0073]

30   Antibody isotypes were determined by antigen-dependent ELISA using isotype-specific secondary antibodies.   hMpl-Fc was diluted to 1 µg/mL with a coating buffer (0.1 mM NaHCO₃, pH9.6) containing 0.02% (w/v) NaN₃, and then added to ELISA plates.   The plates were incubated overnight at 4°C for coating.   The plates were blocked with a diluent buffer (50 mM Tris-HCl (pH8.1) containing 1 mM MgCl₂, 150 mM NaCl, 0.05% (v/v) Tween 20, 0.02% (w/v)

35   NaN₃, 1% (w/v) BSA).   After the addition of hybridoma culture supernatants, the plates were allowed to stand at room temperature for 1 hr.   After washing with a rinse buffer (0.05% (v/v) Tween 20 in PBS), alkaline phosphatase-labeled isotype-specific secondary antibodies were

added to the plates.   Then, the plates were allowed to stand at room temperature for 1 hr.
Color development was carried out using SIGMA104 (Sigma-Aldrich) diluted to 1 mg/mL with
a substrate buffer (50 mM $NaHCO_3$, pH9.8) containing 10 mM $MgCl_2$, and absorbance was
measured at 405 nm using Benchmark Plus (Bio-Rad).

5   [0074]

The binding activities of an antibody to shMpl-FLAG and hMPL-Fc were determined
by ELISA.   ELISA plates were coated with 1 μg/mL of purified shMpl-FLAG or hMPL-Fc, and
blocked with a diluent buffer.   Hybridoma culture supernatants were added to the plates, and the
plates were allowed to stand at room temperature for 1 hr.   Then, alkaline phosphatase-labeled
10   anti-mouse IgG antibodies (Zymed) were added to the plates.   Color development was similarly
carried out using the above method.   Following a one-hour coloring reaction at room
temperature, absorbance was measured at 405 nm and EC_{50} values were computed using
GraphPad Prism.

[0075]

15   CHO-human Mpl cells and CHO-monkey Mpl cells were harvested, and suspended in
FACS Buffer (1% FBS/ PBS) to a final concentration of 1×10^6 cells/mL.   The suspensions
were aliquoted into Multiscreen (Millipore) at 100 μl/well, and the culture supernatants were
removed by centrifugation.   Culture supernatants diluted to 5 μg/mL were added to the plates
and incubated on ice for 30 min.   The cells were washed once with FACS buffer, and incubated
20   on ice for 30 min following the addition of an FITC-labeled anti-mouse IgG antibody (Beckman
Coulter).   After incubation, the mixture was centrifuged at 500 rpm for 1 min.   The
supernatants were removed, and then the cells were suspended in 400 μL of FACS buffer.   The
samples were analyzed by flow cytometry using EPICS ELITE ESP (Beckman Coulter).   An
analysis gate was set on the forward and side scatters of a histogram to include viable cell
25   populations.

[0076]

Agonistic activities of an antibody were evaluated using BaF3-human Mpl and
BaF3-monkey Mpl which proliferate in a TPO-dependent manner.   Cells of each cell line were
suspended at 4×10^5 cells/ml in RPMI 1640/10% FBS (Invitrogen), and each suspension was
30   aliquoted into a 96-well plate at 60μl/well.   A 40 μL aliquot of rhTPO (R&D) and hybridoma
culture supernatants prepared at various concentrations was added into each well.   The plates
were then incubated at 37°C under 5% CO_2 for 24 hr.   A 10-μL aliquot of the Cell Count
Reagent SF (Nacalai Tesque) was added into each well.   After incubation for 2 hr, absorbance
was measured at 450 nm (and at 655 nm as a control) using a Benchmark Plus. EC_{50} values
35   were calculated using GraphPad Prism.

[0077]

The above analysis yielded human Mpl-binding antibodies, VB 22B, VB16, VB140,

VB45B, and TA 136.

[0078]

1.7 Purification of anti-human Mpl antibodies

Anti-human Mpl antibodies were purified from hybridoma culture supernatants as described below.   After the culture supernatants were loaded onto HiTrap protein G HP columns (Amersham Biosciences) for adsorption, the antibodies were eluted with 0.1 M glycine-HCl (pH2.7) Buffer.   Immediately after elution, the fractions were neutralized with 1 M Tris-HCl Buffer (pH9.0), and dialyzed against PBS to replace the buffer for one day.

[0079]

[Example 2]   Preparation of single-chain anti-human Mpl antibodies

Examples for preparing single-chain antibodies from the VB22B anti-human Mpl antibody are described below.

2.1 Cloning of the anti-human Mpl antibody variable region

The variable region was amplified by RT-PCR using total RNA extracted from hybridomas producing anti-human Mpl antibodies.   Total RNA was extracted from 1×10^7 hybridoma cells using the RNeasy Plant Mini Kit (QIAGEN).

[0080]

A 5'-terminal fragment of the gene was amplified from 1 μg of total RNA by the SMART RACE cDNA Amplification Kit (Clontech), using a synthetic oligonucleotide MHC-IgG2b (SEQ ID NO: 3) complementary to mouse IgG2b constant region or a synthetic oligonucleotide kappa (SEQ ID NO: 4) complementary to mouse κ chain constant region. Reverse transcription was carried out at 42°C for 1.5 hr.

The composition of the PCR reaction solution (50 μL in total) is shown below.

10x Advantage 2 PCR Buffer (Clontech)	5 μL
10x Universal Primer A Mix (Clontech)	5 μL
dNTPs (dATP, dGTP, dCTP, and dTTP) (Clontech)	0.2 mM
Advantage 2 Polymerase Mix (Clontech)	1 μL
Reverse transcription product	2.5 μL
Synthetic oligonucleotide, MHC-IgG2b or kappa	10 pmole

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 5 sec and 72°C for 3 min;

five cycles of 94°C for 5 sec, 70°C for 10 sec, and 72°C for 3 min;

25 cycles of 94°C for 5 sec, 68°C for 10 sec, and 72°C for 3 min; and final extension was at 72°C for 7 min.

[0081]

The PCR products were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and cloned into a pGEM-T Easy Vector (Promega). The nucleotide sequence was then determined using the ABI 3700 DNA Analyzer (Perkin Elmer). The nucleotide sequence of cloned VB22B H chain variable region (hereinafter abbreviated as "VB22B-VH") is
5   shown in SEQ ID NO: 5, and the amino acid sequence encoded thereby is shown in SEQ ID NO: 6. The nucleotide sequence of the L chain variable region (hereinafter abbreviated as "VB22B-VL") is shown in SEQ ID NO: 7, and the amino acid sequence encoded thereby is shown in SEQ ID NO: 8. The amino acid sequences of VB22B, VB16, VB140, VB45B, and TA136 are shown in Fig. 1.
10   [0082]

2.2 Preparation of expression vectors for anti-human Mpl diabodies

A gene encoding a VB22B single-chain Fv (hereinafter abbreviated as "VB22B diabody") containing a five-amino acid linker sequence was constructed, by linking a nucleotide sequence encoding a $(Gly_4Ser)_1$ linker to the VB22B-VH-encoding gene at its 3' end and to the
15   VB22B-VL-encoding gene at its 5' end; both genes had been amplified by PCR.
[0083]

The VB22B-VH forward primer, 70·115HF, (SEQ ID NO: 9) was designed to contain an EcoRI site. The VB22B-VH reverse primer, 33·115HR, (SEQ ID NO: 10) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence
20   encoding the $(Gly_4Ser)_1$ linker and a nucleotide sequence hybridizing to the DNA encoding the N terminus of VB22B-VL. The VB22B-VL forward primer, 33·115LF, (SEQ ID NO: 11) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the $(Gly_4Ser)_1$ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH. The VB22B-VL reverse primer, 33·115LR, (SEQ ID NO: 12) was designed to
25   hybridize to a DNA encoding the C terminus of VB22B-VL and to have a nucleotide sequence encoding a FLAG tag (Asp Tyr Lys Asp Asp Asp Asp Lys/SEQ ID NO: 13) and a NotI site.
[0084]

In the first round of PCR, two PCR products: one containing VB22B-VH and a linker sequence, and the other containing VB22B-VL and the identical linker sequence, were
30   synthesized by the procedure described below.

The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pGEM-T Easy vector comprising VB22B-VH or VB22B-VL gene	10 ng
Synthetic oligonucleotides, 70·115HF and 33·115HR, or 33·115LF and 33·115LR	10 pmole

The PCR reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of: 94°C for 15 sec and 72°C for 2 min;

5   five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0085]

After the PCR products of about 400 bp were purified from agarose gel using the

10   QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots

of the respective PCR products according to the protocol described below.

The composition of the PCR reaction solution (50 μL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 unit
First-round PCR products (two types)	1 μL
Synthetic oligonucleotides, 70·115HF and 33·115LR	10 pmole

The reaction conditions were:

15   94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

20   [0086]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick

Gel Extraction Kit (QIAGEN), and then digested with EcoRI and NotI (both from TaKaRa).

The resulting DNA fragments were purified using the QIAquick PCR Purification Kit

(QIAGEN), and then cloned into pCXND3 to prepare pCXND3-VB22B db.

25   [0087]

2.3 Preparation of expression vectors for anti-human Mpl antibody sc(Fv)2

To prepare expression plasmids for the modified antibody [sc(Fv)2] comprising two

units of H chain variable region and two units of L chain variable region derived from VB22B,

the above-described pCXND3-VB22B db was modified by PCR using the procedure shown

30   below.   The process for constructing the sc(Fv)2 gene is illustrated in Fig. 2.

[0088]

First, PCR method was carried out to amplify (a) the VB22B-VH-encoding gene in

which a nucleotide sequence encoding a 15-amino acid linker (Gly$_4$Ser)$_3$ was added to its 3' end; and (b) the VB22B-VL-encoding gene containing the identical linker nucleotide sequence added to its 5' end.   The desired construct was prepared by linking these amplified genes.   Three new primers were designed in this construction process.   The VB22B-VH forward primer,

5   VB22B-fpvu, (primer A; SEQ ID NO: 14) was designed to have an EcoRI site at its 5' end and to convert Gln22 and Leu23 of VB22B db into a PvuII site.   The VB22B-VH reverse primer, sc-rL15, (primer B; SEQ ID NO: 15) was designed to hybridize to a DNA encoding the C terminus of VB22B-VH, and to have a nucleotide sequence encoding the (Gly$_4$Ser)$_3$ linker, as well as a nucleotide sequence hybridizing to a DNA encoding the N terminus of VB22B-VL.

10   The VB22B-VL forward primer, sc-fL15, (primer C; SEQ ID NO: 16) was designed to have a nucleotide sequence encoding the N terminus of VB22B-VL, a nucleotide sequence encoding the (Gly$_4$Ser)$_3$ linker, and a nucleotide sequence encoding the C terminus of VB22B-VH.
[0089]

       In the first-round PCR, two PCR products: one comprising VB22B-VH and a linker

15   sequence, and the other comprising VB22B-VL and the identical linker sequence, were synthesized by the procedure described below.

       The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pCXND3-VB22B db	10 ng
Synthetic oligonucleotides, VB22B-fpvu, sc-rL15 or sc-fL15, and 33·115LR (primer D)	10 pmole

       The reaction conditions were:

20   94°C (initial temperature) for 30 sec;
     five cycles of 94°C for 15 sec and 72°C for 2 min;
     five cycles of 94°C for 15 sec and 70°C for 2 min;
     28 cycles of 94°C for 15 sec and 68°C for 2 min;
     and final extension was at 72°C for 5 min.

25   [0090]

       After the PCR products of about 400 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), the second-round PCR was carried out using aliquots of the respective PCR products according to the protocol described below.

       The composition of the PCR reaction solution (50 µL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 µL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM

DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
First-round PCR product (two types)	1 μL
Synthetic oligonucleotide, 70·115HF and 33·115LR	10 pmole

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

5   five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

[0091]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick

10   Gel Extraction Kit (QIAGEN), and then digested with EcoRI and NotI (both from TaKaRa).
The resulting DNA fragments were purified using the QIAquick PCR Purification Kit
(QIAGEN), and then cloned into pBacPAK9 (Clontech) to construct pBacPAK9-scVB22B.

[0092]

A fragment to be inserted into the PvuII site of pBacPAK9-scVB22B was prepared.

15   Specifically, the fragment has a PvuII recognition site at both ends and a nucleotide sequence, in
which a gene encoding the VB22B-VH N-terminus is linked, via a (Gly4Ser)3 linker-encoding
nucleotide sequence, to a gene encoding the amino acid sequence of an N terminus-deleted
VB22B-VH linked to VB22B-VL via the (Gly4Ser)3 linker.   Two primers were newly designed
to prepare the fragment by PCR.   The forward primer for the fragment of interest, Fv2-f (primer

20   E; SEQ ID NO: 17), was designed to have a PvuII site at its 5' end and a VB22B-VH 5'-end
sequence.   The reverse primer for the fragment of interest, Fv2-r (primer F; SEQ ID NO: 18),
was designed to hybridize to a DNA encoding the C terminus of VB22B-VL, and to have a PvuII
site, a nucleotide sequence encoding the (Gly4Ser)3 linker, and a nucleotide sequence hybridizing
to a DNA encoding the N terminus of VB22B-VH.   PCR was carried out using

25   pBacPAK9-scVB22B as a template as described below.

The composition of the PCR reaction solution (50 μL in total) is shown below.

10x PCR Buffer (TaKaRa)	5 μL
dNTPs (dATP, dGTP, dCTP, and dTTP) (TaKaRa)	0.4 mM
DNA polymerase TaKaRa Ex Taq (TaKaRa)	2.5 units
pBacPAK9-scVB22B	10 μg
Synthetic oligonucleotide, Fv2-f and Fv2-r	10 pmole

The reaction conditions were:

94°C (initial temperature) for 30 sec;

five cycles of 94°C for 15 sec and 72°C for 2 min;

five cycles of 94°C for 15 sec and 70°C for 2 min;

28 cycles of 94°C for 15 sec and 68°C for 2 min;

and final extension was at 72°C for 5 min.

5 [0093]

The PCR products of about 800 bp were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN), and then cloned into the pGEM-T Easy Vector (Promega). After sequencing, the plasmid was digested with PvuII (TaKaRa), and the fragment of interest was recovered. The recovered fragment was ligated to pBacPAK9-scVB22B pre-digested with

10 PvuII (TaKaRa) to construct pBacPAK9-VB22B sc(Fv)2. After the resulting vector was digested with EcoRI and NotI (both from TaKaRa), the fragment of about 1,800 bp was purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN). The fragment was then cloned into a pCXND3 expression vector to construct pCXND3-VB22B sc(Fv)2.

[0094]

15 2.4 Expression of single-chain anti-human Mpl antibody in animal cells

A cell line stably expressing the single-chain antibody was prepared from CHO-DG44 cells as described below. Gene transfer was achieved by electroporation using a Gene Pulser II (Bio-Rad). An expression vector (25 μg) and 0.75 mL of CHO-DG44 cells suspended in PBS $(1 \times 10^7$ cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred

20 into a cuvette, and pulsed at 1.5-kV and 25 μFD. After a ten-minute restoration period at room temperature, the electroporated cells were plated in CHO-S-SFMII medium (Invitrogen) containing 500 μg/mL Geneticin (Invitrogen). CHO cell lines expressing the single-chain antibody were established through selection. A cell line stably expressing VB22B sc(Fv)2 and its culture supernatants were obtained by this method.

25 [0095]

The transient expression of the single-chain antibody was achieved using COS7 cells as described below. An expression vector (10 μg) and 0.75 mL of COS7 cells suspended in PBS $(1 \times 10^7$ cells/mL) were mixed. The resulting mixture was cooled on ice for 10 min, transferred into a cuvette, and then pulsed at 1.5-kV and 25 μFD. After a ten-minute restoration period at

30 room temperature, the electroporated cells were plated in DMEM/10% FBS medium (Invitrogen). The cells were incubated overnight and then washed with PBS. CHO-S-SFMII medium was added and the cells were cultured for about three days. The culture supernatants for preparing the VB22B diabody were thus prepared.

[0096]

35 2.5 Quantitation of single-chain anti-human Mpl antibodies in culture supernatants

The culture supernatant concentration of the single-chain anti-human Mpl antibody transiently expressed in COS7 cells or CHO cells was determined using surface plasmon

resonance.   A sensor chip CM5 (Biacore) was placed in BIAcore 2000 (Biacore).
ANTI-FLAG® M2 Monoclonal Antibody (Sigma-Aldrich) was immobilized onto the chip.   An
appropriate concentration of sample was injected over the chip surface at a flow rate of 5 mL/sec,
and 50 mM diethylamine was used to dissociate the bound antibody.   Changes in the mass

5   during sample injection were recorded, and the sample concentration was calculated from the
calibration curve prepared using the mass changes of a standard sample.   db12E10 (see
Japanese Patent Application No. 2001-27734) was used as the diabody standard, and 12E10
sc(Fv)2 which has the same gene structure as that of sc(Fv)2 was used as the sc(Fv)2 standard.
[0097]

10   2.6 Purification of anti-human Mpl single-chain antibodies
        The culture supernatants of VB22B diabody-expressing COS7 cells or CHO cells was
loaded onto an Anti-Flag M2 Affinity Gel (Sigma-Aldrich) column equilibrated with a 50 mM
Tris-HCl buffer (pH7.4) containing 150 mM NaCl and 0.05% Tween 20.   The absorbed
antibodies were eluted with 100 mM glycine-HCl (pH3.5).   The fractions eluted were

15   immediately neutralized with 1 M Tris-HCl (pH8.0), and loaded onto a HiLoad 26/60 Superdex
200 pg (Amersham Biosciences) column for gel filtration chromatography.   PBS/0.01% Tween
20 was used in the gel filtration chromatography.
[0098]
        VB22B sc(Fv)2 was purified from the culture supernatants of VB22B

20   sc(Fv)2-expressing COS7 cells or CHO cells under the same conditions used for purifying the
diabodies.   A large-scale preparation of VB22B sc(Fv)2 was prepared by loading the CHO cell
culture supernatants onto a Macro-Prep Ceramic Hydroxyapatite Type I (Bio-Rad) column
equilibrated with a 20 mM phosphate buffer (pH6.8), and eluting the VB22B sc(Fv)2 in a
stepwise manner with 250 mM phosphate buffer (pH6.8).   The eluted fraction was concentrated

25   on an ultrafilter, and then fractionated by gel filtration chromatography using a HiLoad 26/60
Superdex 200 pg (Amersham Biosciences) column, and a fraction corresponding to the
molecular weight range of about 40 kD to 70 kD was obtained.   The fraction was loaded onto
an Anti-Flag M2 Affinity Gel column equilibrated with a 50 mM Tris-HCl buffer (pH7.4)
containing 150 mM NaCl and 0.05% Tween 20.   The absorbed antibody was eluted with 100

30   mM glycine-HCl (pH3.5).   The eluted fraction was immediately neutralized with 1 M Tris-HCl
(pH8.0), and loaded onto a HiLoad 26/60 Superdex 200 pg (Amersham Biosciences) column for
gel filtration chromatography.   20 mM acetate (pH6.0) containing 150 mM NaCl and 0.01%
Tween 80 was used in the gel filtration chromatography.
[0099]

35   2.7 Assessment of TPO-like agonist activity for single-chain anti-human Mpl antibodies
        TPO-like agonist activity was assessed using BaF3-human Mpl that proliferate in a
TPO-dependent manner.   The cells were washed twice with RPMI 1640/1% FBS (fetal bovine

serum) (Invitrogen), and then suspended in RPMI 1640/10% FBS to a concentration of 4×10^5 cells/mL.   Cell suspensions were aliquoted at 60 μL/well into a 96-well plate.   Various concentrations of rhTPO (R&D) and COS7 culture supernatants or purified samples were prepared, and a 40 μL aliquot was added into each well.   The plates were then incubated at

5   37°C under 5% CO_2 for 24 hr.   Immediately after a 10-μL aliquot of WST-8 reagent (Cell Count Reagent SF; Nacalai Tesque) was added into each well, absorbance was measured at 450 nm (and at 655 nm as a control) using Benchmark Plus.   After two hours of incubation, absorbance was again measured at 450 nm (and at 655 nm as a control). The WST-8 reagent changes colors at 450 nm in a color reaction that reflects the viable cell count.   The TPO-like

10   agonist activity was assessed using the change in absorbance during the two-hour incubation as an index. EC_{50} values were computed using GraphPad Prism.

[0100]

The results of assessing the TPO-like agonist activity of the purified VB22B diabody and VB22B sc(Fv)2 are shown in Fig. 3 (BaF3-human Mpl) and Fig. 4 (BaF3-monkey Mpl).

15   Furthermore, the single-chain antibodies (diabody and sc(Fv)2) of VB16 (Fig. 5), VB140 (Fig. 6), VB45B (Fig. 7), and TA136 (Fig. 8) are expressed in COS7 cells and the culture supernatants were used to assess the antibodies' TPO-like agonist activity in BaF3-human Mpl.   In addition, the EC_{50} values obtained from the analyses described above are shown in Table 1.

[0101]

20

[Table 1]

The agonistic activities (EC$_{50}$ value: pM) of antibodies VB22B, VB16, VB140, VB45B, and TA136 in BaF-human Mpl and BaF-monkey Mpl.

Antibody	BaF-human Mpl		BaF-monkey Mpl	
	Diabody	sc(Fv)2	Diabody	sc(Fv)2
VB22B	61	27	1668	26
VB16	190	95		
VB140	89	38		
VB45B	76	30		
TA136	3076	54		

5   [0102]

These results confirmed that the sc(Fv)2 single-chain antibodies exhibit a higher agonistic activity than the diabodies.   For the agonistic activity, it is essential that the antigen binding site is divalent.   The distance and angle between two antigen binding sites are also considered to be important factors (see Japanese Patent Application No. 2001-277314).   The

10   optimal distance and angle vary depending on the epitope recognized by each of the antibodies obtained.   The optimal linker length differs from antibody to antibody.   However, it has been reported that when the linker length is as short as 5-12mer, a non-covalent diabody is formed; and when the linker length is longer (12mer or longer), a scFv monomer is formed instead of a diabody (Hudson *et al.*, J Immunol. Methods 1999, 231:177-189).   Thus, it can be predicted

15   that sc(Fv)2, in which a divalent antigen-binding site is formed regardless of the use of a long linker, is very likely to have a high agonistic activity.   In addition, since the sc(Fv)2 which is linked by a linker is more stable, there is a possibility that it can confer a higher activity as compared with a non-covalent diabody.

[Brief Description of the Drawings]

20   [0103]

Fig. 1 shows the amino acid sequence of an anti-human Mpl antibody (H and L chains). The H chain amino acid sequences of VB140, VB45B, VB22B, VB16, and TA136 are shown as SEQ ID NOs: 19, 20, 21, 22, and 23, respectively.

Further, the L chain amino acid sequences of VB140, VB45B, VB22B, VB16, and TA136 are

25   shown in SEQ ID NOs: 24, 25, 26, 27, and 28 respectively.

Fig. 2 shows the process for constructing a single-chain antibody, sc(Fv)2.

Fig. 3 illustrates the result of evaluating VB22B antibody's agonistic activity using BaF-human Mpl.

Fig. 4 illustrates the result of evaluating VB22B antibody's agonistic activity using BaF-monkey Mpl.

Fig. 5 illustrates the result of evaluating VB16 antibody's agonistic activity using BaF-human Mpl.

5   Fig. 6 illustrates the result of evaluating VB140 antibody's agonistic activity using BaF-human Mpl.

Fig. 7 illustrates the result of evaluating VB45B antibody's agonistic activity using BaF-human Mpl.

Fig. 8 illustrates the result of evaluating TA136 antibody's agonistic activity using

10   BaF-human Mpl.

[Sequence Listing]

```
<110>  CHUGAI SEIYAKU KABUSHIKI KAISHA


<120>  Methods for enhancing activity of antibody.
15
<130>  C1-A0321


<160>  28


20    <170>  PatentIn version 3.1


<210>  1
<211>  1924
<212>  DNA
25    <213>  Macaca fascicularis


<220>
<221>  CDS
<222>  (11)..(1918)
30    <223>


<400>  1
gaattccacc atg ccc tcc tgg gcc ctc ttc atg gtc acc tcc tgc ctc        49
           Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu
           1               5                   10
35

ctc ctg gcc cct caa aac ctg gcc caa gtc agc agc caa gat gtc tcc        97
```

```
    Leu Leu Ala Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser
         15              20                25
```

```
     ttg ctg gcc tcg gac tca gag ccc ctg aag tgt ttc tcc cga aca ttt     145
  5  Leu Leu Ala Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe
     30              35                40                45
```

```
     gag gac ctc act tgc ttc tgg gat gag gaa gag gca gca ccc agt ggg     193
     Glu Asp Leu Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly
 10              50                55                60
```

```
     aca tac cag ctg ctg tat gcc tac ccg ggg gag aag ccc cgt gcc tgc     241
     Thr Tyr Gln Leu Leu Tyr Ala Tyr Pro Gly Glu Lys Pro Arg Ala Cys
                 65                70                75
 15
     ccc ctg agt tct cag agc gtg ccc cgc ttt gga acc cga tac gtg tgc     289
     Pro Leu Ser Ser Gln Ser Val Pro Arg Phe Gly Thr Arg Tyr Val Cys
                 80                85                90
```

```
 20  cag ttt cca gcc cag gaa gaa gtg cgt ctc ttc tct ccg ctg cac ctc     337
     Gln Phe Pro Ala Gln Glu Glu Val Arg Leu Phe Ser Pro Leu His Leu
                 95                100               105
```

```
     tgg gtg aag aat gtg ttc cta aac cag act cag att cag cga gtc ctc     385
 25  Trp Val Lys Asn Val Phe Leu Asn Gln Thr Gln Ile Gln Arg Val Leu
     110             115               120               125
```

```
     ttt gtg gac agt gta ggc ctg ccg gct ccc ccc agt atc atc aag gcc     433
     Phe Val Asp Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala
 30                  130               135               140
```

```
     atg ggt ggg agc cag cca ggg gaa ctt cag atc agc tgg gag gcc cca     481
     Met Gly Gly Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro
                 145               150               155
 35
     gct cca gaa atc agt gat ttc ctg agg tac gaa ctc cgc tat ggc ccc     529
     Ala Pro Glu Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro
```

```
                160                    165                    170

       aaa gat ctc aag aac tcc act ggt ccc acg gtc ata cag ttg atc gcc    577
       Lys Asp Leu Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala
 5         175                    180                    185


       aca gaa acc tgc tgc cct gct ctg cag agg cca cac tca gcc tct gct    625
       Thr Glu Thr Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala
           190                    195                    200                    205
10
       ctg gac cag tct cca tgt gct cag ccc aca atg ccc tgg caa gat gga    673
       Leu Asp Gln Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly
                              210                    215                    220


15     cca aag cag acc tcc cca act aga gaa gct tca gct ctg aca gca gtg    721
       Pro Lys Gln Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val
                              225                    230                    235


       ggt gga agc tgc ctc atc tca gga ctc cag cct ggc aac tcc tac tgg    769
20     Gly Gly Ser Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp
                  240                    245                    250


       ctg cag ctg cgc agc gaa cct gat ggg atc tcc ctc ggt ggc tcc tgg    817
       Leu Gln Leu Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp
25         255                    260                    265


       gga tcc tgg tcc ctc cct gtg act gtg gac ctg cct gga gat gca gtg    865
       Gly Ser Trp Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val
       270                    275                    280                    285
30
       gca att gga ctg caa tgc ttt acc ttg gac ctg aag aat gtt acc tgt    913
       Ala Ile Gly Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys
                              290                    295                    300


35     caa tgg cag caa gag gac cat gct agt tcc caa ggt ttc ttc tac cac    961
       Gln Trp Gln Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His
                              305                    310                    315
```

```
     agc agg gca cgg tgc tgc ccc aga gac agg tac ccc atc tgg gag gac      1009
     Ser Arg Ala Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp
             320             325             330
 5
     tgt gaa gag gaa gag aaa aca aat cca gga tta cag acc cca cag ttc      1057
     Cys Glu Glu Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe
             335             340             345

10   tct cgc tgc cac ttc aag tca cga aat gac agc gtt att cac atc ctt      1105
     Ser Arg Cys His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu
     350             355             360             365

     gtg gag gtg acc aca gcc ctg ggt gct gtt cac agt tac ctg ggc tcc      1153
15   Val Glu Val Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser
                 370             375             380

     cct ttc tgg atc cac cag gct gtg cgc ctc ccc acc cca aac ttg cac      1201
     Pro Phe Trp Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His
20                   385             390             395

     tgg agg gag atc tcc agc ggg cat ctg gaa ttg gag tgg cag cac cca      1249
     Trp Arg Glu Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro
                 400             405             410
25
     tca tcc tgg gca gcc caa gag acc tgc tat caa ctc cga tac aca gga      1297
     Ser Ser Trp Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly
             415             420             425

30   gaa ggc cat cag gac tgg aag gtg ctg gag ccg cct ctc ggg gcc cga      1345
     Glu Gly His Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg
     430             435             440             445

     gga ggg acc ctg gag ctg cgc ccg cga tct cgc tac cgt tta cag ctg      1393
35   Gly Gly Thr Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu
                 450             455             460
```

```
      cgc gcc agg ctc aat ggc ccc acc tac caa ggt ccc tgg agc tcg tgg       1441
      Arg Ala Arg Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp
                      465             470             475

 5    tcg gac cca gct agg gtg gag acc gcc acc gag acc gcc tgg att tcc       1489
      Ser Asp Pro Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser
                  480             485             490

      ttg gtg acc gct ctg ctg cta gtg ctg ggc ctc agc gcc gtc ctg ggc       1537
10    Leu Val Thr Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly
                  495             500             505

      ctg ctg ctg ctg agg tgg cag ttt cct gca cac tac agg aga ctg agg       1585
      Leu Leu Leu Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg
15    510             515             520             525

      cat gcc ctg tgg ccc tca ctt cca gat ctg cac cga gtc cta ggc cag       1633
      His Ala Leu Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln
                      530             535             540
20
      tac ctt agg gac act gca gcc ctg agt ccg ccc aag gcc aca gtc tca       1681
      Tyr Leu Arg Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser
                      545             550             555

25    gat acc tgt gaa gaa gtg gaa ccc agc ctc ctt gaa atc ctc ccc aag       1729
      Asp Thr Cys Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys
                  560             565             570

      tcc tca gag agg act cct ttg ccc ctg tgt tcc tcc cag tcc cag atg       1777
30    Ser Ser Glu Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met
                  575             580             585

      gac tac cga aga ttg cag cct tct tgc ctg ggg acc atg ccc ctg tct       1825
      Asp Tyr Arg Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser
35    590             595             600             605

      gtg tgc cca ccc atg gct gag tca ggg tcc tgc tgt acc acc cac att       1873
```

```
        Val Cys Pro Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile
                        610                 615                 620


        gcc aac cat tcc tac cta cca cta agc tat tgg cag cag cct tga        1918
    5   Ala Asn His Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
                        625                 630                 635


        gtcgac                                                             1924


    10  <210>   2
        <211>   635
        <212>   PRT
        <213>   Macaca fascicularis


    15  <400>   2
        Met Pro Ser Trp Ala Leu Phe Met Val Thr Ser Cys Leu Leu Leu Ala
        1               5                   10                  15


        Pro Gln Asn Leu Ala Gln Val Ser Ser Gln Asp Val Ser Leu Leu Ala
    20                  20                  25                  30


        Ser Asp Ser Glu Pro Leu Lys Cys Phe Ser Arg Thr Phe Glu Asp Leu
                        35                  40                  45


    25  Thr Cys Phe Trp Asp Glu Glu Glu Ala Ala Pro Ser Gly Thr Tyr Gln
                50                  55                  60


        Leu Leu Tyr Ala Tyr Pro Gly Glu Lys Pro Arg Ala Cys Pro Leu Ser
        65                  70                  75                  80
    30
        Ser Gln Ser Val Pro Arg Phe Gly Thr Arg Tyr Val Cys Gln Phe Pro
                        85                  90                  95


        Ala Gln Glu Glu Val Arg Leu Phe Ser Pro Leu His Leu Trp Val Lys
    35                  100                 105                 110


        Asn Val Phe Leu Asn Gln Thr Gln Ile Gln Arg Val Leu Phe Val Asp
```

```
                115                    120                      125

        Ser Val Gly Leu Pro Ala Pro Pro Ser Ile Ile Lys Ala Met Gly Gly
             130                    135                140
  5
        Ser Gln Pro Gly Glu Leu Gln Ile Ser Trp Glu Ala Pro Ala Pro Glu
        145                 150                155                160

        Ile Ser Asp Phe Leu Arg Tyr Glu Leu Arg Tyr Gly Pro Lys Asp Leu
 10                       165                170                175

        Lys Asn Ser Thr Gly Pro Thr Val Ile Gln Leu Ile Ala Thr Glu Thr
                          180                185                190

 15     Cys Cys Pro Ala Leu Gln Arg Pro His Ser Ala Ser Ala Leu Asp Gln
                      195                 200                205

        Ser Pro Cys Ala Gln Pro Thr Met Pro Trp Gln Asp Gly Pro Lys Gln
                 210                 215                220
 20
        Thr Ser Pro Thr Arg Glu Ala Ser Ala Leu Thr Ala Val Gly Gly Ser
        225                 230                235                240

        Cys Leu Ile Ser Gly Leu Gln Pro Gly Asn Ser Tyr Trp Leu Gln Leu
 25                       245                250                255

        Arg Ser Glu Pro Asp Gly Ile Ser Leu Gly Gly Ser Trp Gly Ser Trp
                     260                 265                270

 30     Ser Leu Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Ala Ile Gly
                 275                 280                285

        Leu Gln Cys Phe Thr Leu Asp Leu Lys Asn Val Thr Cys Gln Trp Gln
                 290                 295                300
 35
        Gln Glu Asp His Ala Ser Ser Gln Gly Phe Phe Tyr His Ser Arg Ala
        305                 310                315                320
```

```
         Arg Cys Cys Pro Arg Asp Arg Tyr Pro Ile Trp Glu Asp Cys Glu Glu
                     325             330             335

 5       Glu Glu Lys Thr Asn Pro Gly Leu Gln Thr Pro Gln Phe Ser Arg Cys
                     340             345             350

         His Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu Val Glu Val
                     355             360             365
10
         Thr Thr Ala Leu Gly Ala Val His Ser Tyr Leu Gly Ser Pro Phe Trp
                 370             375             380

         Ile His Gln Ala Val Arg Leu Pro Thr Pro Asn Leu His Trp Arg Glu
15       385             390             395             400

         Ile Ser Ser Gly His Leu Glu Leu Glu Trp Gln His Pro Ser Ser Trp
                         405             410             415

20       Ala Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly His
                     420             425             430

         Gln Asp Trp Lys Val Leu Glu Pro Pro Leu Gly Ala Arg Gly Gly Thr
                     435             440             445
25
         Leu Glu Leu Arg Pro Arg Ser Arg Tyr Arg Leu Gln Leu Arg Ala Arg
                 450             455             460

         Leu Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ser Trp Ser Asp Pro
30       465             470             475             480

         Ala Arg Val Glu Thr Ala Thr Glu Thr Ala Trp Ile Ser Leu Val Thr
                     485             490             495

35       Ala Leu Leu Leu Val Leu Gly Leu Ser Ala Val Leu Gly Leu Leu Leu
                 500             505             510
```

```
     Leu Arg Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu
             515                 520                 525


     Trp Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg
  5      530                 535                 540


     Asp Thr Ala Ala Leu Ser Pro Pro Lys Ala Thr Val Ser Asp Thr Cys
     545                 550                 555                 560


 10  Glu Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu
                 565                 570                 575


     Arg Thr Pro Leu Pro Leu Cys Ser Ser Gln Ser Gln Met Asp Tyr Arg
                 580                 585                 590

 15
     Arg Leu Gln Pro Ser Cys Leu Gly Thr Met Pro Leu Ser Val Cys Pro
                 595                 600                 605


     Pro Met Ala Glu Ser Gly Ser Cys Cys Thr Thr His Ile Ala Asn His
 20      610                 615                 620


     Ser Tyr Leu Pro Leu Ser Tyr Trp Gln Gln Pro
     625                 630                 635


 25  <210>  3
     <211>  24
     <212>  DNA
     <213>  Artificial


 30  <220>
     <223>  an artificially synthesized sequence


     <400>  3
     cagggggccag tggatagact gatg                                           24
 35
     <210>  4
     <211>  23
```

40

```
    <212>  DNA
    <213>  Artificial

    <220>
5   <223>  an artificially synthesized sequence

    <400>  4
    gctcactgga tggtgggaag atg                                          23


10  <210>  5
    <211>  411
    <212>  DNA
    <213>  Mus musculus

15  <220>
    <221>  CDS
    <222>  (1)..(411)
    <223>

20  <400>  5
    atg gaa tgg cct ttg atc ttt ctc ttc ctc ctg tca gga act gca ggt    48
    Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
    1               5                   10                  15

25  gtc cac tcc cag gtt cag ctg cag cag tct gga cct gag ctg gtg aag    96
    Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
                    20                  25                  30

    cct ggg gcc tca gtg aag att tcc tgc aag gct tct ggc tat gca ttc   144
30  Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
                35                  40                  45

    act aac tcc tgg atg aac tgg gtg aag cag agg cct gga aag ggt ctt   192
    Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
35      50                  55                  60

    gag tgg att gga cgg att tat cct gga gat gga gaa act atc tac aat   240
```

Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn
65   70   75   80

<div>

```
      ggg aaa ttc agg gtc aag gcc aca ctg act gca gac aaa tcc tcc agc        288
  5   Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
                      85                  90                  95

      aca gcc tac atg gat atc agc agc ctg aca tct gag gac tct gcg gtc        336
      Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
 10                       100             105             110

      tac ttc tgt gca aga ggc tat gat gat tac tcg ttt gct tac tgg ggc        384
      Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
                  115             120             125
 15
      caa ggg act ctg gtc act gtc tct gca                                    411
      Gln Gly Thr Leu Val Thr Val Ser Ala
          130             135
```

</div>

```
 20   <210>  6
      <211>  137
      <212>  PRT
      <213>  Mus musculus

 25   <400>  6
      Met Glu Trp Pro Leu Ile Phe Leu Phe Leu Leu Ser Gly Thr Ala Gly
      1               5                   10                  15

      Val His Ser Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys
 30                   20                  25                  30

      Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe
                  35                  40                  45

 35   Thr Asn Ser Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu
                  50                  55                  60
```

```
      Glu Trp Ile Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn
      65              70              75              80


      Gly Lys Phe Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser
5                     85              90              95


      Thr Ala Tyr Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val
                      100             105              110


10    Tyr Phe Cys Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly
                  115             120             125


      Gln Gly Thr Leu Val Thr Val Ser Ala
          130             135
15
      <210>  7
      <211>  396
      <212>  DNA
      <213>  Mus musculus
20
      <220>
      <221>  CDS
      <222>  (1)..(396)
      <223>
25
      <400>  7
      atg agg tgc cta gct gag ttc ctg ggg ctg ctt gtg ttc tgg att cct       48
      Met Arg Cys Leu Ala Glu Phe Leu Gly Leu Leu Val Phe Trp Ile Pro
      1               5               10              15
30
      gga gcc att ggg gat att gtg atg act cag gct gca ccc tct ata cct       96
      Gly Ala Ile Gly Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro
                      20              25              30


35    gtc act cct gga gag tca gta tcc atc tcc tgt agg tct agt aag agt      144
      Val Thr Pro Gly Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser
                      35              40              45
```

```
       ctc ctg cat agt aat ggc aac act tac ttg tat tgg ttc ctg cag agg        192
       Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg
            50              55              60
 5
       cca ggc cag tct cct caa ctc ctg ata tat cgg atg tcc aac ctt gcc        240
       Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala
       65              70              75              80

10     tca gga gtc cca gat agg ttc agt ggc agt ggg tca gga act gct ttc        288
       Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe
                       85              90              95

       aca ctg aga atc agt aga gtg gag gct gag gat gtg ggt gtt tat tac        336
15     Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr
                    100             105             110

       tgt atg caa cat ata gaa tat cct ttt acg ttc gga tcg ggg acc aag        384
       Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys
20              115             120             125

       ctg gaa ata aaa                                                        396
       Leu Glu Ile Lys
            130
25
       <210>  8
       <211>  132
       <212>  PRT
       <213>  Mus musculus
30
       <400>  8
       Met Arg Cys Leu Ala Glu Phe Leu Gly Leu Leu Val Phe Trp Ile Pro
       1               5               10              15

35     Gly Ala Ile Gly Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro
                       20              25              30
```

44

```
        Val Thr Pro Gly Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser
                35                      40                  45

        Leu Leu His Ser Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg
   5        50                  55                  60

        Pro Gly Gln Ser Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala
        65                  70                  75                  80

  10    Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe
                        85                  90                  95

        Thr Leu Arg Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr
                    100                 105                 110
  15
        Cys Met Gln His Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys
                    115                 120                 125

        Leu Glu Ile Lys
  20            130

        <210>  9
        <211>  30
        <212>  DNA
  25    <213>  Artificial

        <220>
        <223>  an artificially synthesized primer sequence

  30    <400>  9
        tagaattcca ccatggaatg gcctttgatc                                    30

        <210>  10
        <211>  56
  35    <212>  DNA
        <213>  Artificial
```

<220>
<223>   an artificially synthesized primer sequence

<400>   10
agcctgagtc atcacaatat ccgatccgcc tccacctgca gagacagtga ccagag   56

<210>   11
<211>   56
<212>   DNA
<213>   Artificial

<220>
<223>   an artificially synthesized primer sequence

<400>   11
actctggtca ctgtctctgc aggtggaggc ggatcggata ttgtgatgac tcaggc   56

<210>   12
<211>   60
<212>   DNA
<213>   Artificial

<220>
<223>   an artificially synthesized primer sequence

<400>   12
attgcggccg cttatcactt atcgtcgtca tccttgtagt cttttatttc cagcttggtc   60

<210>   13
<211>   8
<212>   PRT
<213>   Artificial

<220>
<223>   an artificially synthesized FLAG tag sequence

<400>   13

```
Asp Tyr Lys Asp Asp Asp Asp Lys
1                   5
```

```
     <210>  14
5    <211>  85
     <212>  DNA
     <213>  Artificial


     <220>
10   <223>  an artificially synthesized primer sequence


     <400>  14
     tagaattcca ccatggaatg gcctttgatc tttctcttcc tcctgtcagg aactgcaggt     60


15   gtccactccc aggttcagct gcagc                                           85


     <210>  15
     <211>  82
     <212>  DNA
20   <213>  Artificial


     <220>
     <223>  an artificially synthesized primer sequence


25   <400>  15
     tgagtcatca caatatccga tccgccacca cccgaaccac caccacccga accaccacca     60


     cctgcagaga cagtgaccag ag                                              82


30   <210>  16
     <211>  82
     <212>  DNA
     <213>  Artificial


35   <220>
     <223>  an artificially synthesized primer sequence
```

```
<400>  16
tggtcactgt ctctgcaggt ggtggtggtt cgggtggtgg tggttcgggt ggtggcggat    60


cggatattgt gatgactcag gc                                            82
```

```
<210>  17
<211>  25
<212>  DNA
<213>  Artificial


<220>
<223>  an artificially synthesized primer sequence


<400>  17
caggttcagc tgcagcagtc tggac                                         25


<210>  18
<211>  81
<212>  DNA
<213>  Artificial


<220>
<223>  an artificially synthesized primer sequence


<400>  18
gctgcagctg aacctgcgat ccaccgcctc ccgaaccacc accacccgat ccaccacctc    60


cttttatttc cagcttggtc c                                             81


<210>  19
<211>  118
<212>  PRT
<213>  Mus musculus


<400>  19
Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
1               5                   10                  15
```

```
            Ser Val Lys Ile Ser Cys Arg Ala Phe Gly Tyr Ala Phe Ser Asn Ser
                        20                  25                  30

    5       Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                        35                  40                  45

            Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe
                        50              55                  60
   10
            Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
            65                  70                  75                  80

            Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
   15                       85                  90                  95

            Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                            100                 105                 110

   20       Leu Val Thr Val Ser Ala
                            115


            <210>   20
            <211>   118
   25       <212>   PRT
            <213>   Mus musculus


            <400>   20
            Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
   30       1               5                   10                  15


            Ser Val Lys Ile Ser Cys Arg Ala Phe Gly Tyr Ala Phe Ser Asn Ser
                        20                  25                  30


   35       Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                        35                  40                  45
```

```
        Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Asn Asn Asn Gly Lys Phe
            50              55              60

        Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
     5  65              70              75              80

        Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
                        85              90                  95

    10  Ala Arg Gly Tyr Gly Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                    100             105             110

        Leu Val Thr Val Ser Ala
                115
    15
        <210>  21
        <211>  118
        <212>  PRT
        <213>  Mus musculus
    20
        <400>  21
        Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
        1               5               10              15

    25  Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ala Phe Thr Asn Ser
                    20              25              30

        Trp Met Asn Trp Val Lys Gln Arg Pro Gly Lys Gly Leu Glu Trp Ile
                    35              40              45
    30
        Gly Arg Ile Tyr Pro Gly Asp Gly Glu Thr Ile Tyr Asn Gly Lys Phe
            50              55              60

        Arg Val Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr
    35  65              70              75              80

        Met Asp Ile Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Phe Cys
```

50

```
                    85                      90                      95

        Ala Arg Gly Tyr Asp Asp Tyr Ser Phe Ala Tyr Trp Gly Gln Gly Thr
                        100                 105                 110
5
        Leu Val Thr Val Ser Ala
                        115


        <210>   22
10      <211>   115
        <212>   PRT
        <213>   Mus musculus


        <400>   22
15      Gln Val Gln Leu Gln Gln Pro Gly Thr Glu Leu Val Arg Pro Gly Ala
        1                   5                   10                  15

        Ser Val Lys Leu Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
                        20                  25                  30
20
        Trp Val Asn Trp Val Lys Gln Arg Pro Gly Arg Gly Leu Glu Trp Ile
                        35                  40                  45

        Gly Arg Ile His Pro Tyr Asp Ser Glu Thr His Tyr Asn Gln Lys Phe
25          50                  55                  60

        Lys Asn Lys Ala Thr Leu Thr Val Asp Lys Ser Ser Ser Thr Ala Tyr
        65                  70                  75                  80

30      Ile Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
                        85                  90                  95

        Ala Ser Gly Gly Trp Phe Ala Ser Trp Gly Gln Gly Thr Leu Val Thr
                        100                 105                 110
35
        Val Ser Ala
                        115
```

```
      <210>  23
      <211>  116
      <212>  PRT
  5   <213>  Mus musculus

      <400>  23
      Asp Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
      1               5                   10                  15
 10
      Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile Thr Ser Asp
                      20                  25                  30

      Tyr Ala Trp Ser Trp Ile Arg Gln Leu Pro Gly Asn Lys Leu Glu Trp
 15           35                  40                  45

      Met Gly Tyr Ile Thr Tyr Ser Gly Tyr Ser Ile Tyr Asn Pro Ser Leu
              50                  55                  60

 20   Lys Ser Arg Ile Ser Ile Ser Arg Asp Thr Ser Lys Asn Gln Leu Phe
      65                  70                  75                  80

      Leu Gln Leu Asn Ser Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys
                      85                  90                  95
 25
      Val Gly Gly Tyr Asp Asn Met Asp Tyr Trp Gly Gln Gly Thr Ser Val
                      100                 105                 110

      Thr Val Ser Ser
 30                   115


      <210>  24
      <211>  112
      <212>  PRT
 35   <213>  Mus musculus

      <400>  24
```

```
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5               10              15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                20              25              30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35              40              45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
        50              55              60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile
65              70              75              80

Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85              90              95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
            100             105             110
```

```
<210>   25
<211>   112
<212>   PRT
<213>   Mus musculus

<400>   25
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5               10              15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                20              25              30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
            35              40              45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
```

```
                    50                      55                      60


        Asp Arg Phe Ser Gly Ser Gly Ser Gly Ala Ala Phe Thr Leu Arg Ile
        65                  70                  75                  80

        Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                        85                  90                  95

        Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
                        100                 105                 110


        <210>  26
        <211>  112
        <212>  PRT
        <213>  Mus musculus


        <400>  26
        Asp Ile Val Met Thr Gln Ala Ala Pro Ser Ile Pro Val Thr Pro Gly
        1               5                   10                  15


        Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu His Ser
                        20                  25                  30


        Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
                    35                  40                  45


        Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
                50                  55                  60


        Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Arg Ile
        65                  70                  75                  80


        Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                        85                  90                  95


        Ile Glu Tyr Pro Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
                        100                 105                 110
```

```
<210>  27
<211>  112
<212>  PRT
<213>  Mus musculus

<400>  27
Asp Ile Val Met Thr Gln Ala Ala Pro Ser Val Pro Val Thr Pro Gly
1               5                   10                  15

Glu Ser Val Ser Ile Ser Cys Arg Ser Ser Lys Ser Leu Leu Tyr Ser
            20                  25                  30

Asn Gly Asn Thr Tyr Leu Tyr Trp Phe Leu Gln Arg Pro Gly Gln Ser
        35                  40                  45

Pro Gln Leu Leu Ile Tyr Arg Met Ser Asn Leu Ala Ser Gly Val Pro
    50                  55                  60

Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Ala Phe Thr Leu Thr Ile
65                  70                  75                  80

Ser Ser Val Glu Ala Glu Asp Val Gly Val Tyr Tyr Cys Met Gln His
                85                  90                  95

Leu Glu Tyr Pro Tyr Thr Phe Gly Ser Gly Thr Lys Leu Glu Ile Lys
                100                 105                 110


<210>  28
<211>  108
<212>  PRT
<213>  Mus musculus

<400>  28
Gln Ile Val Leu Thr Gln Ser Pro Ala Ile Met Ser Ala Ser Pro Gly
1               5                   10                  15
```

```
        Glu Lys Val Thr Leu Thr Cys Ser Ala Ser Ser Ser Val Ser Ser Ser
                     20                  25                  30

        His Leu Tyr Trp Tyr Gln Gln Lys Pro Gly Ser Ser Pro Lys Leu Trp
   5                 35                  40                  45

        Ile Tyr Ser Thr Ser Asn Leu Ala Ser Gly Val Pro Ala Arg Phe Ser
                     50                  55                  60

  10    Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser Asn Met Glu
        65                  70                  75                  80

        Thr Glu Asp Ala Ala Ser Tyr Phe Cys His Gln Trp Ser Ser Tyr Pro
                         85                  90                  95

  15
        Trp Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
                        100                 105
```

[Document Name] Drawings

[Fig.1]

<H CHAIN>

		CDR1		CDR2
VB140	QVQLQQSGPELVKPGASVKISCRAFGYAFS	NSWMN	WVKQRPGKGLEWIG	RIYPGDGETNNNGKFKG
VB45B	QVQLQQSGPELVKPGASVKISCKASGYAFS	SSWMN	WVKQRPGKGLEWIG	RIYPGDGETNNNGKFKG
VB22B	QVQLQQSGPELVKPGASVKISCKASGYAFT	NSWMN	WVKQRPGKGLEWIG	RIYPGDGETIYNGKFRV
VB16	QVQLQQPGTELVRPGASVKLSCKASGYTFT	DYWVN	WVKQRPGRGLEWIG	RIHPYDSETHYNQKFKN
TA136	DVQLQESGPGLVKPSQSLSLTCTVTGYSIT	SDYAWS	WIRQLPGNKLEWMG	YITYSGYSI-YNPSLKS

		CDR3	
VB140	KATLTADKSSSTAYMQLSSLTSEDSAVYFCAR	GYGD--YSFAY	WGQGTLVTVSA
VB45B	KATLTADKSSTTAYMQLSSLTSEDSAVYFCAR	GYGD--YSFAY	WGQGTLVTVSA
VB22B	KATLTADKSSSTAYMDISSLTSEDSAVYFCAR	GYDD--YSFAY	WGQGTLVTVSA
VB16	KATLTVDKSSSTAYIQLSSLTSEDSAVYYCAS	GGW-----FAS	WGQGTLVTVSA
TA136	RISISRDTSKNQLFLQLNSVTTEDTATYYCVG	GYDNMD----Y	WGQGTSVTVSS

<L CHAIN>

		CDR1		CDR2
VB140	DIVMTQAAPSVPVTPGESVSISC	RSSKSLLHSNGNTYLY	WFLQRPGQSPQLLIY	RMSNLAS
VB45B	DIVMTQAAPSVPVTPGESVSISC	RSSKSLLHSNGNTYLY	WFLQRPGQSPQLLIY	RMSNLAS
VB22B	DIVMTQAAPSIPVTPGESVSISC	RSSKSLLHSNGNTYLY	WFLQRPGQSPQLLIY	RMSNLAS
VB16	DIVMTQAAPSVPVTPGESVSISC	RSSKSLLYSNGNTYLY	WFLQRPGQSPQLLIY	RMSNLAS
TA136	QIVLTQSPAIMSASPGEKVTLTC	SASSSVSSSH----LY	WYQQKPGSSPKLWIY	STSNLAS

|		CDR3	
VB140	GVPDRFSGSGSGAAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB45B	GVPDRFSGSGSGAAFTLRISRVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
VB22B	GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC	MQHIEYPFT	FGSGTKLEIK
VB16	GVPDRFSGSGSGTAFTLTISSVEAEDVGVYYC	MQHLEYPYT	FGSGTKLEIK
TA136	GVPARFSGSGSGTSYSLTISNMETEDAASYFC	HQWSSYPWT	FGGGTKLEIK

[Fig.2]

[Fig.3]

[Fig.4]

5

[Fig.5]

6
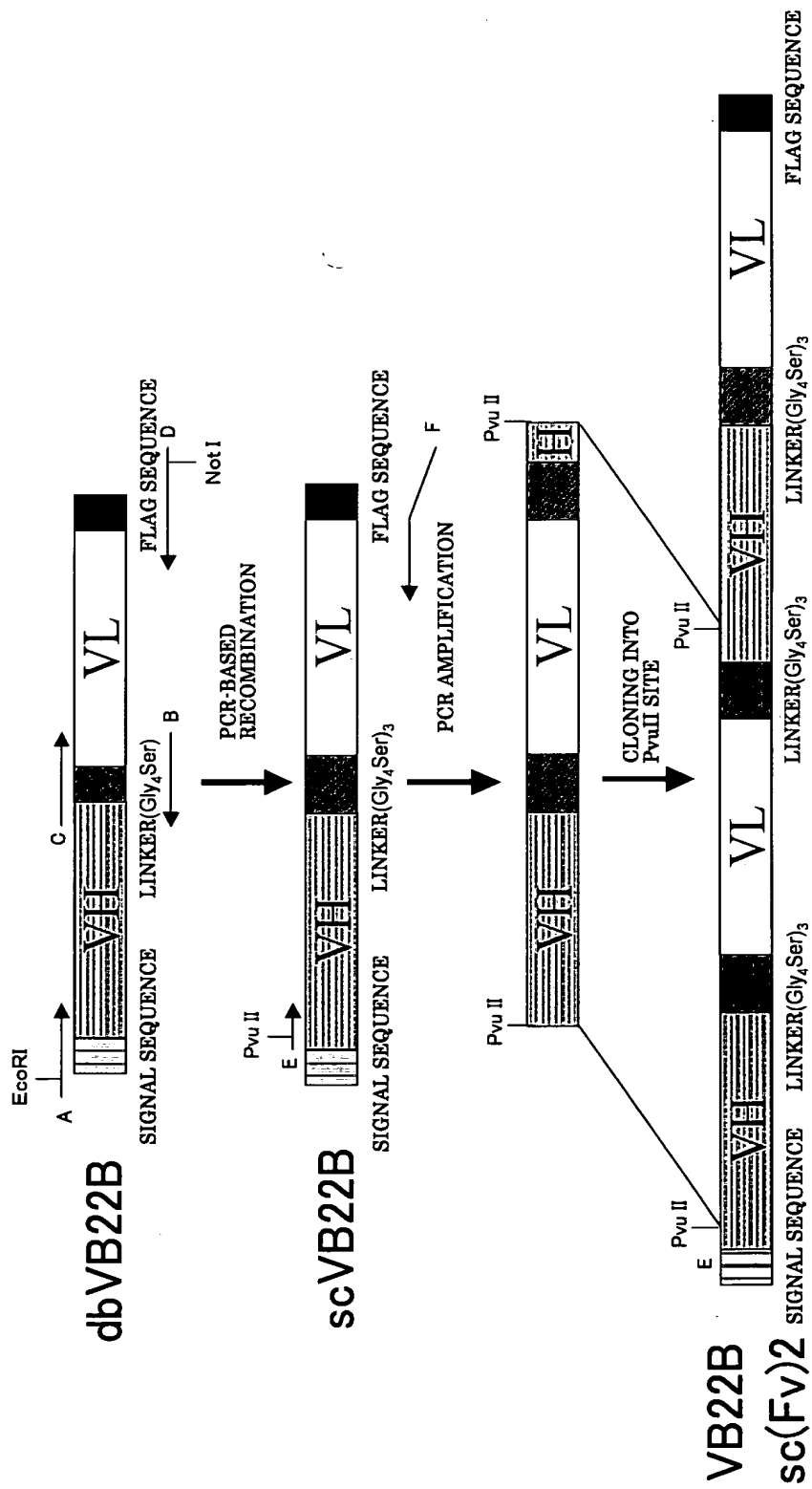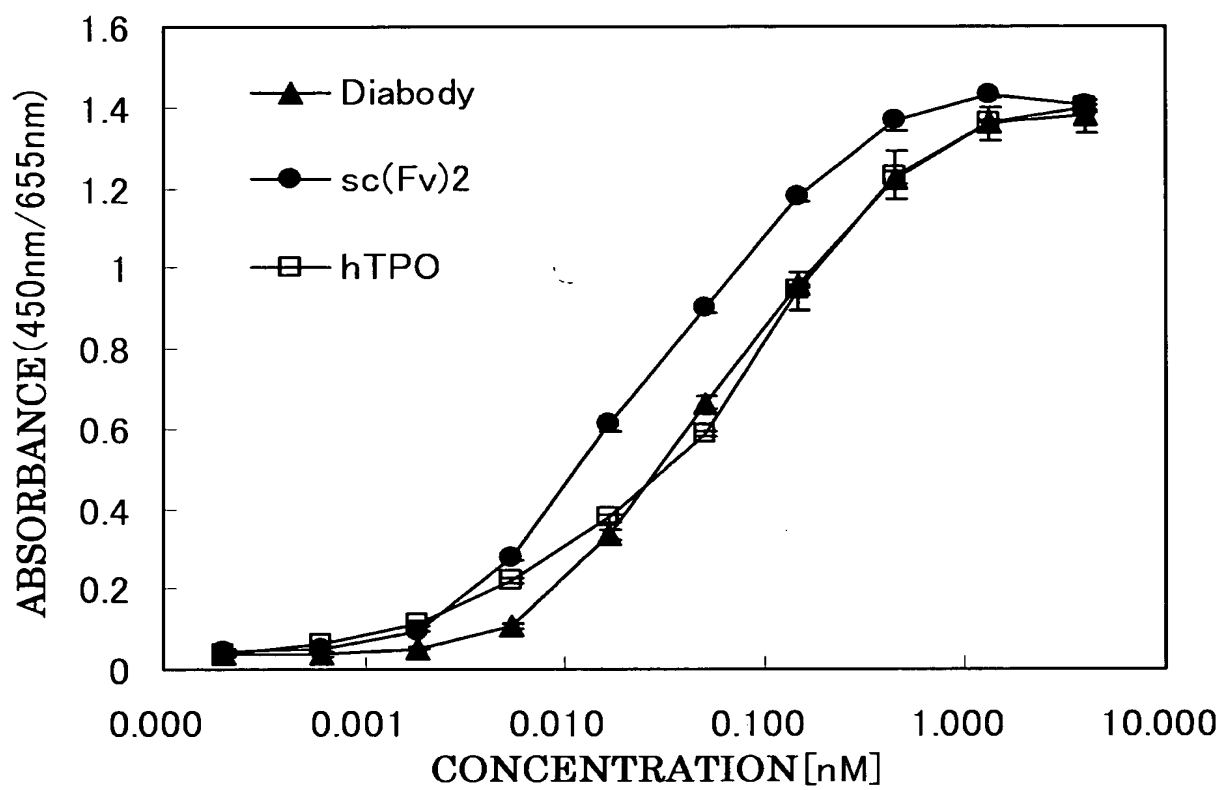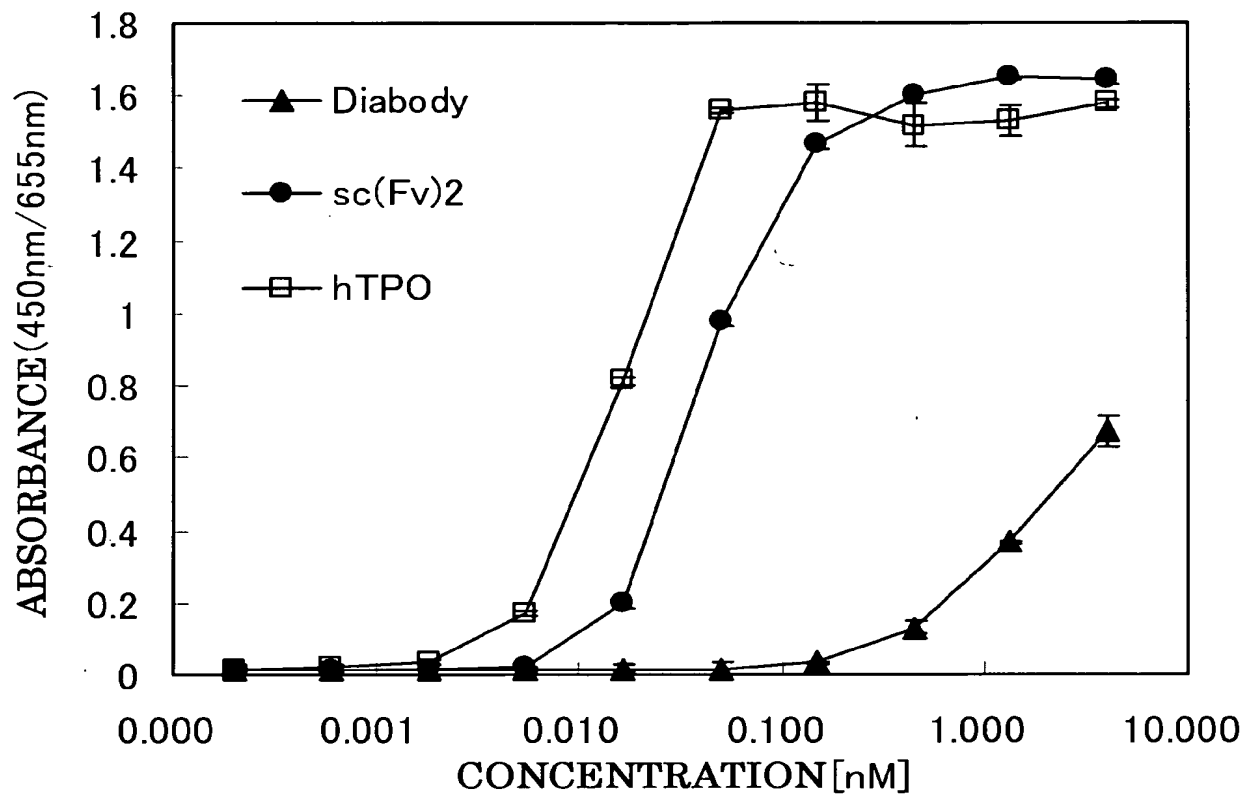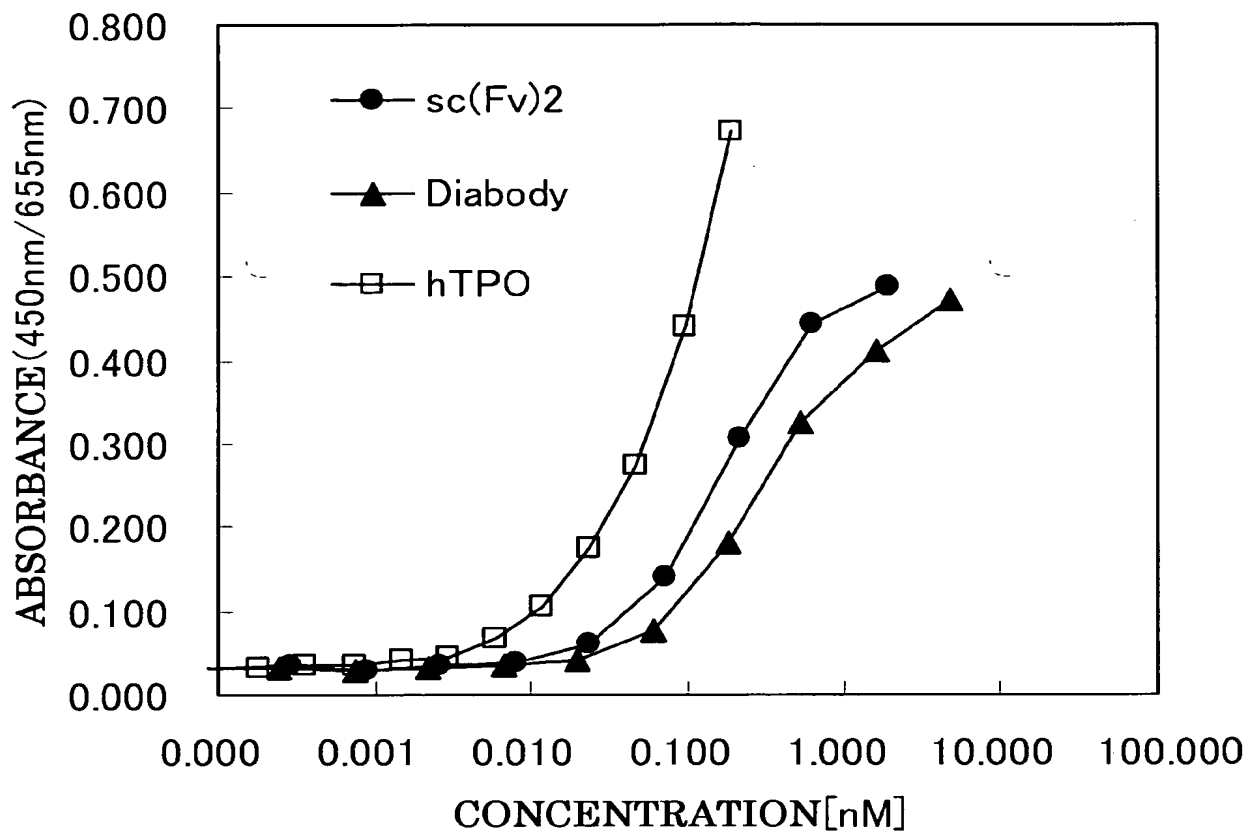
[Fig.6]
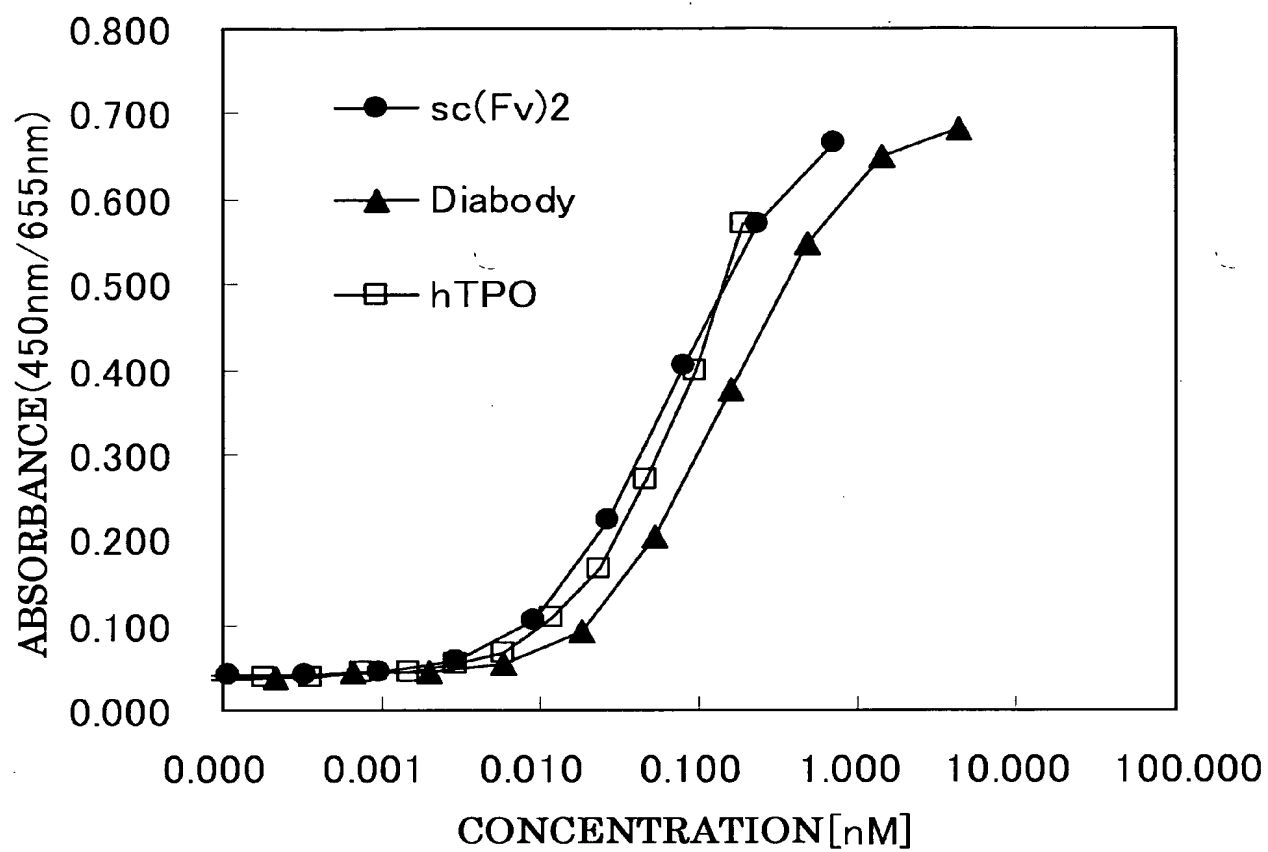
[Fig.7]
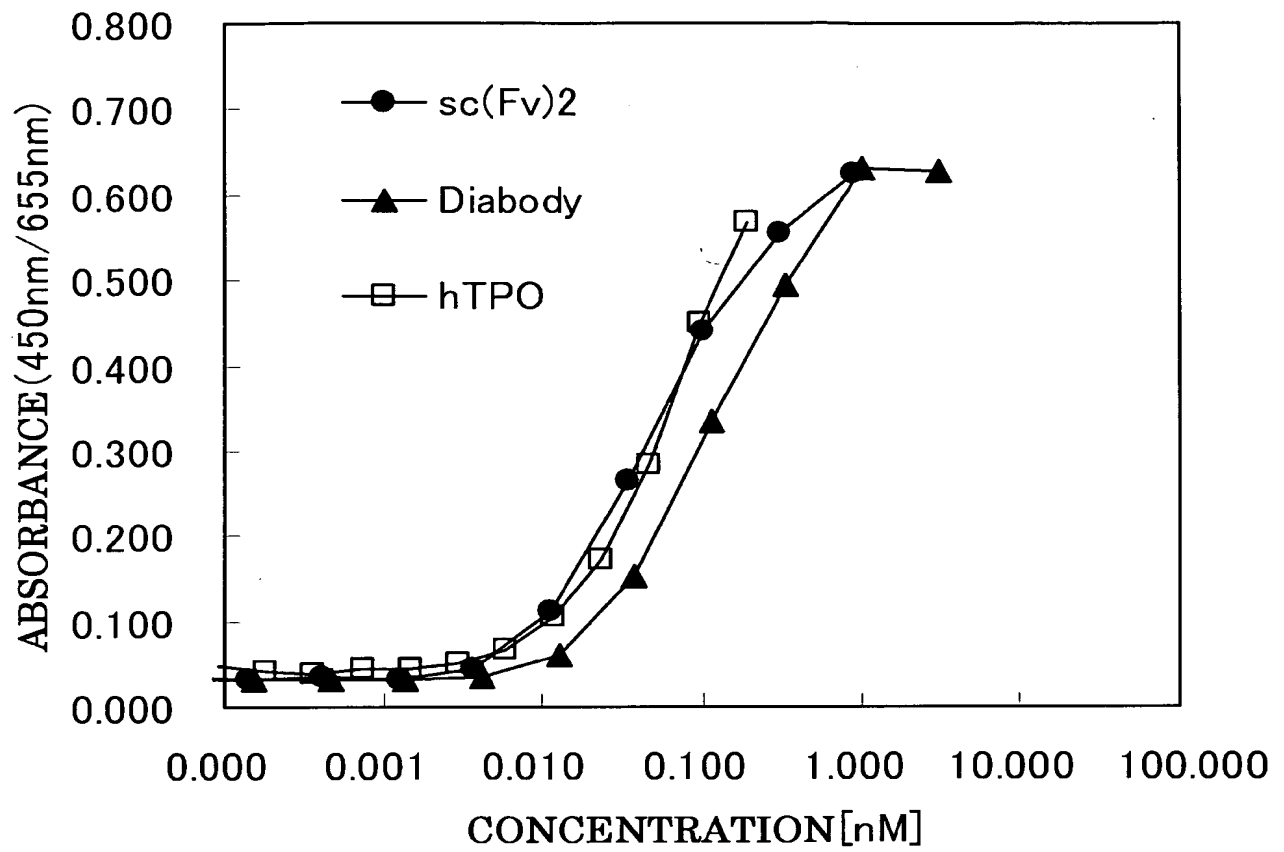
[Fig.8]
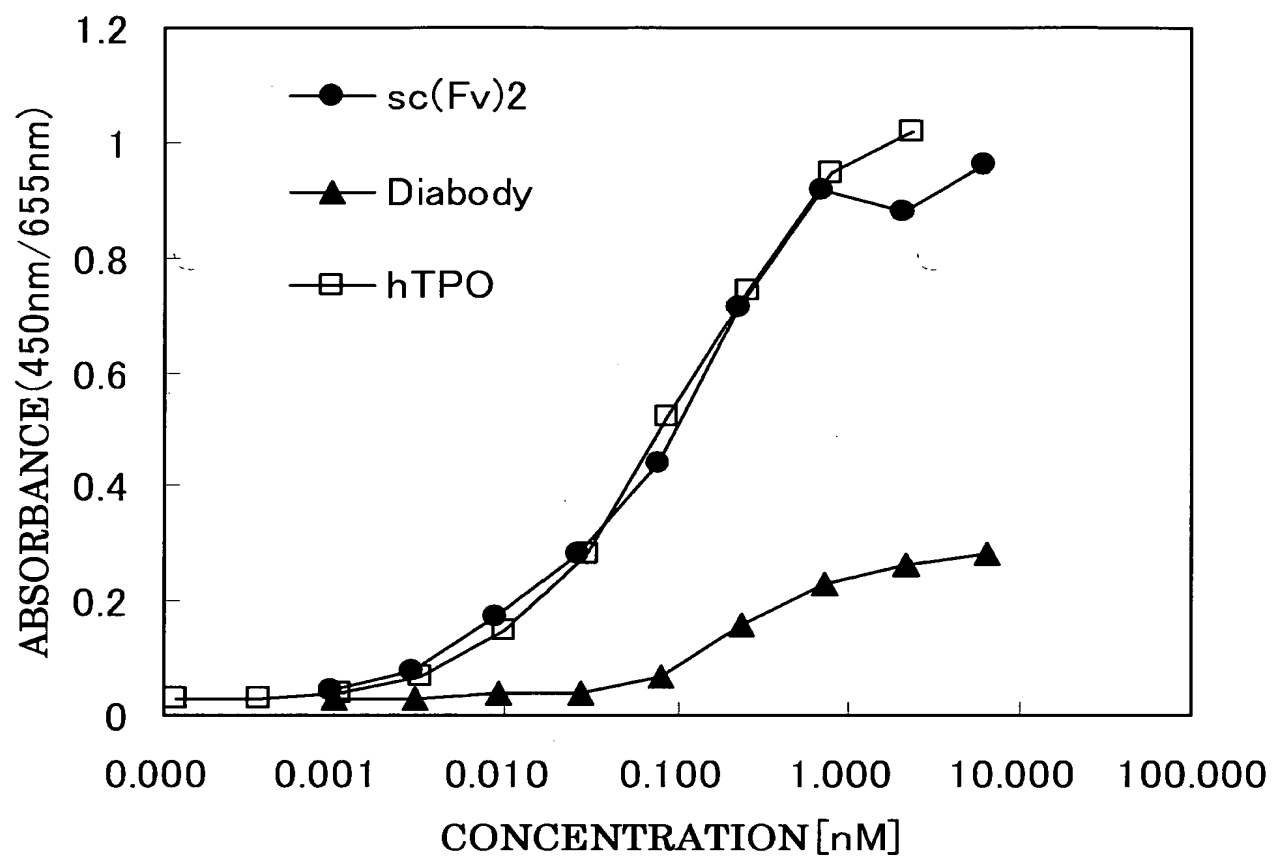
[Document Name] Abstract

[Abstract]

[Problems to Be Solved]

5   The objective of the present invention is to provide methods for enhancing antibody activity, which comprise preparing a single-chain polypeptide in which two or more light chain variable regions are linked to two or more heavy chain variable regions via linkers.

[Means to Solve the Problem]

        Anti-human Mpl antibodies were isolated and purified, and then single-chain

10   anti-human Mpl antibodies were prepared using genetic engineering techniques.   The antibodies were found to exhibit a high agonistic activity.   This shows that the activity of an antibody can be enhanced by making the antibody into a single-chain polypeptide that comprises two or more heavy chain variable regions and two or more light chain variable regions linked via linkers.

15   [Selected Drawings] None